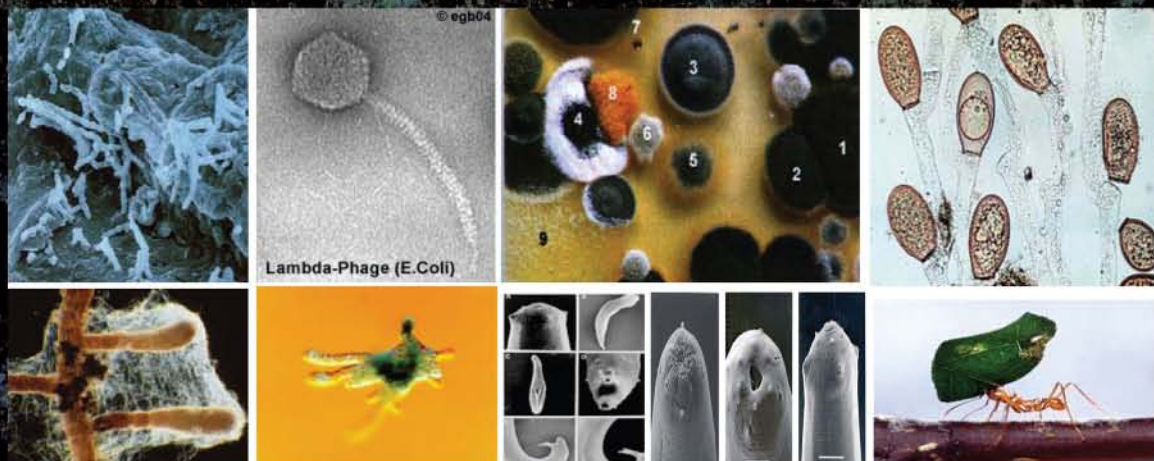


Biodiversity in Agricultural Production Systems

Edited by
Gero Benckiser
Sylvia Schnell

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Preface

Many industries are based on nature's products: food processing, cosmetics, pharmaceuticals, and pulp and paper, to name but a few. Thus, a judicious use of biodiversity in agriculture or wastewater treatment is essential both for the maintenance of our life-support system and for the sustainable development of our world's resources. Human activity has been identified as one of the primary drivers of changes in biodiversity. There are three areas for action:

1. intensive production systems such as agriculture, livestock, aquaculture and tree plantations where priority is given to their life-support functions, the maintenance of genetic biodiversity, and where caution is exercised in admitting alien invasive species;
2. production systems involving wild species such as forestry, wildlife, and fisheries where the focus should be on maintaining an array of productive ecosystems; and
3. habitats in protected areas, where stronger links are needed between conservation action and sustainable development strategies.

Agricultural biodiversity, especially at agroecosystem levels (area 1) on which this book is focused, is determined by the genetic resources, the physical environment, and the human management practices, and has spatial and temporal scale dimensions. Effective solutions for a sustainable management lay in understanding the microbial, faunal, and plant individuals and/or communities and how they work together.

Most of the Earth's terrestrial species live in the soil. These organisms, which include many thousands of species of bacteria, fungi, protozoa, nematodes, and other soil animals, shape above-ground plant and animal life as well as our climate and atmosphere. It thus encompasses the variety and variability of animals, plants, and microorganisms necessary to sustain key functions of the agroecosystem, its structure, and processes for, and in support of, food production and food security (FAO, 1999). Up to now, above- and below-ground ecology and agricultural practice have been conducted largely in isolation. But there has been considerable progress in understanding the more proximate mechanisms generating yield stability and changes, such as changes in nutrient availability, habitat fragmentation, pollution, invasive species, as well as the effects of such changes on ecosystem processes. Incorporating this information into strategies that provide incentives for the sustainable use of biodiversity requires an integrative approach. In addition to the Marcel Dekker publication *Fauna in Soil Ecosystems*, which discusses the importance of fauna for agricultural production, *Biodiversity in Agricultural Production Systems* aims to work with an interdisciplinary community of like-minded researchers of above- and below-ground interactions for the welfare of high-yielding, sustainable agriculture.

Accordingly, *Biodiversity in Agricultural Production Systems* aims to:

1. describe the diversity of agricultural production systems, particularly geno/phenotype diversity through plant breeding;
2. collect data on soil space diversity and dynamics;
3. analyze species richness of microbial communities (bacteria, fungi, protozoa, nematodes) in soils and in plant interactions as well as those of tardigrades;
4. focus on lumbricid earthworms and ants as important distributors or microbes in soils;
5. describe the metabolic diversity of microorganisms combined with distribution and functions of soil enzymes;

6. summarize greenhouse gas emissions through agriculture;
7. outline principles and strategies of order between interacting molecules, cells, species, and communities;
8. model food chain interactions, and
9. discuss biological soil characteristics and long-term field observations for soil quality assessment and sustainability.

Editors

Gero Benckiser is Lecturer in Soil Microbiology at the Institute for Applied Microbiology, Justus Liebig University, Giessen, Germany. He is a member of the International Soil Science Society, the Society of General and Applied Microbiology, and the National Academy of Biological Science of India. For more than 10 years he was chairman of the Soil Biology and Applied Microbiology section of the Association of German Research and Experimental Stations (VDLUFA), and the holder of a German patent. Dr. Benckiser received both the diploma in agrobiolgy (1975) and a doctoral degree in agricultural science (1980) from the University of Stuttgart-Hohenheim, Germany, working in an interdisciplinary waste-water treatment program with denitrifiers growing on recalcitrant substrates such as hexachlorobenzene. Following two-year postdoctoral training, he worked on iron toxicity in rice at the International Rice Research Institute, Los Banos, Philippines. At the Federal Research Center of Agriculture, Braunschweig, Germany, he returned to denitrification measurements in various soil ecosystems. His teaching interests include soil microbiology and food-conservation-related microbiology.

Sylvia Schnell is Professor of General and Soil Microbiology at the Institute for Applied Microbiology, Justus Liebig University, Giessen, Germany, since 2000. She received a diploma in biology in 1989 from the University Konstanz and a Ph.D. in microbiology from the University of Tübingen, working with sulfate reducers degrading aromatic compounds. Following three years of postdoctoral training she worked on methanotrophic bacteria in forest soil at the University of Maine. At the Max Planck Institute for Terrestrial Microbiology in Marburg she started to study microbial iron(III) reduction and iron(II) oxidation in rice paddies. Besides the microbial iron cycle, her recent research also focuses on microbial nitrogen turnover in various habitats. Her teaching interests include microbial ecology, microbial diagnostics, and microbial biotechnology.

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Introduction

Gero Benckiser and Sylvia Schnell

WHAT IS AGRICULTURAL BIODIVERSITY?

The Earth's equilibrium appears to be maintained through ecological processes in which biodiversity plays a central role. Recently the term *agricultural biodiversity* has come into wide use, and virtually there are no ecosystems in the world that are "natural" in the sense of having escaped human influence.^{1,2,3} Agricultural production systems include polycultures, monocultures, mixed crop–live-stock systems, agroforestry, agrosilvopastoral systems, aquaculture, rangelands, pastures, and fallow lands. At the agroecosystem level, spatial, temporal, and scale dimensions are involved (chapter 1 and chapter 3 of this edition). Physicochemical, physiological, and ecological processes lead to a hierarchy of agroecosystems from a field/crop/herd/pond-level to a farming system, a land-use system, or a watershed level.

Agricultural biodiversity encompasses the variety and variability of animals, plants, and micro-organisms, which are necessary for sustained and secure food production. It includes the genetic resources (varieties, breeds, etc.) used for the production of fodder, fiber, fuel, and pharmaceuticals by subsuming the diversity of nonharvested species that support production (e.g., soil microorganisms, predators, pollinators). Furthermore, agricultural biodiversity includes all species in the wider agricultural, pastoral, forest, and aquatic environments that support agroecosystems, and finally it covers the diversity of the agroecosystems themselves, which are maintained by a continuous energy supply in a stable, robust, productive, and sustainable status.

Biodiversity can be characterized by (1) *species richness*, which determines the number of species within an area by giving equal weight to each species, together with (2) *species diversity*, which counts the species in an area by adjusting for sampling effects and species abundance, (3) *taxon diversity*, which measures the taxonomic dispersion of species by taking into account not just numbers of species but taxonomic positions and the contributions that different species make, and (4) *functional diversity*, which assesses the richness of functional features and interrelations in an area by identifying food webs, keystone species, and guilds. The term *functional diversity* subdivides further into (i) *autecology diversity*, which studies single organisms and how they relate to their environments; and (ii) *synecological diversity*, which concentrates on communities and the interrelation of species living together.

Habitat diversity, narrowly applied, refers to diversity of species in the habitat or when loosely applied to gross structural and compositional differences of some community of species in the local environment.

Systems diversity is assessed as the richness of ecological systems in a region or landscape.

Ecological diversity refers to the number of species in a given area and the ecological roles that these species play, and considers also the way that the composition of species changes across a region as well as the groupings of species (ecosystems) that occur in particular areas. Ecological diversity includes the processes and interactions that take place within and between these systems and covers diversity of ecosystems from landscape to planet level.

Biodiversity, finally, is covering all the above diversity scales, and is claimed to be an indicator of sustainability or of a system that survives or persists. It concerns temporality and, in particular, longevity.

AGRICULTURAL ECOSYSTEM STABILITY

The longevity of agricultural production systems is guaranteed by biodiverse key functions. The main key functions are:

- balanced soil organic matter stabilization and nutrient recycling for maintaining soil fertility and an efficient plant and animal growth by moderate greenhouse gas emissions;
- breakdown of applied pollutants for limited side effects on neighboring environments;
- moderation of climatic effects for conserving rainfall patterns, the modulation of the water cycle, and the absorption of solar energy;
- maintenance and stability of productive vegetative, animal, and microbial populations for reducing invasions of harmful or less useful species;
- protection and conservation of soil and water resources through appropriate management practices, and the consequent maintenance of the integrity of landscapes and habitats; and
- sufficient sequestration of CO₂ by plants and microbes for balancing the metabolizing industrial and biological processes.

The environmental and vital subsets as key functions of agricultural production systems provide (1) the food, fuel, and fiber needs of the world's population, (2) regulate the quality of the air and water, (3) decompose organic wastes, (4) recycle nutrients, and (5) act as a sink for pollutants (including global gases). Consequently, habitats with their vital components are described in the following chapters. Resulting from the interaction between the environmental genetic resources and the management systems and practices are the target crops, which were carefully selected together with the accompanying flora and fauna by inventive farmers, herders, and fishers over millennia. Due to the introgression from wild relatives, hybridization between cultivars, mutations, and natural and human selections, valuable ecological and sustainable processes have been manifested, and it is not surprising that farmers' crop varieties or animal breeds are well adapted to local abiotic and biotic environmental variation. Agricultural biodiversity stabilizes for human welfare not only the immediate provision of food and other goods but also the maintenance of areas of land that will finally support the maintenance of the wider environment.

Since the onset of the Green Revolution during the middle of the last century, locally diverse food production systems, together with the accompanying local knowledge, culture, and skills of the food producers, started to change. This change in food production means not only that the agricultural biodiversity of harvested species has been changed but also varieties and breeds of a wide range of unharvested species. Estimates reveal that more than 90% of crop varieties have disappeared from farmers' fields and half of the breeds of many domestic animals have been lost due to rapid expansion of industrial and Green Revolution agriculture. Globalization of the food systems that cultivate relatively few crop varieties in monocultures for a more uniform, less diverse but more competitive global market (some production systems using genetically modified varieties and breeds) influences additionally the rearing of fewer varieties and breeds and extends it to a global scale. Genetic erosion, the loss of genetic diversity, including the loss of individual genes and gene-complexes, is observed worldwide. This development is indeed questionable, because the genes and gene complexes found in the diverse farmers' varieties are not contained in the modern varieties. In China in 1949, nearly 10,000 wheat varieties were used in production. Twenty-one years later, in 1970, about 1,000 varieties remained in use. Together with the decline in wheat varieties, a not-well-studied reduction in the less visible species (invertebrates, microorganisms) will certainly go hand in hand.^{1,2,3} The loss of these underground species could have far-reaching effects on the sustainability of many agricultural production systems around the world, because ecosystem functioning refers to the flow of materials (nutrients, water, atmospheric gases) and the biochemical activities. Microorganisms and invertebrates play a vital role in maintaining and enhancing soil fertility, in detoxifying pesticides and other pollutants, and are involved in the

biological control of agricultural pests.^{1,2,3} In fact, it is the genetic diversity within species that allows them to evolve under changing environments and selection pressures.

Plasticity is the term that describes genetic diversity and flexibility. High genome plasticity depends on mobile genetic elements (plasmids, transposons). Bacteria are furnished with the highest plasticity, because they have the potential to take up easily external genetic information through conjugation, transduction, or transformation and insert it into their own genomes. This capability of high mutation enables bacteria to react quickly on changing environmental conditions by producing new proteins. Gene exchange is recognized both within (horizontal) and between the bacterial clusters (vertical). The output is named species. At present, a bacterial species is defined as composed of organisms that have 97% similarity in their 16S rRNA sequences. Genetic plasticity can diminish immunological defense systems. Genetic plasticity ends up in a phenotypic plasticity, defined as *the environmentally sensitive production of alternative phenotypes by given genotypes*. We are aware of only about 1% of the bacterial phenotypes.⁴ The majority of organisms in the soil are inactive and it is assumed that normally over 90% of the microorganisms are in a resting stage.

The phenotypic plasticity present within a certain area is based on the relative abundance of each species (evenness) and the number of species (richness). It can be described by the equation

$$V_p = V_G + V_E + V_{G \times E} + V_{\text{error}}$$

where V_p is the total phenotypic variance; V_G , the genetic effects; V_E , the environmental effects; $V_{G \times E}$, the genotype–environmental interactions; and V_{error} , the developmental noise.

Strongly intertwined with plasticity is *species redundancy*. The word *redundancy* means in its literal sense unnecessary. If it is true that ecosystem function does not imply purpose or design, only activity, and species redundancy contributes to the regulatory control of ecosystems, then species redundancy is repugnant to defenders of biodiversity.⁵ Agricultural production systems have a basic structure trophically through herbivory, predation, detritivory, or omnivory activities linked to peripheral compartments. They have in common (1) the acquisition of energy by autotrophs (photosynthesis, chemosynthesis); (2) mineralization, or the return of nutrients to inorganic pools by heterotrophs; (3) decomposition, or the transfer of material from dead organic matter to decomposer biomass; (4) immobilization, or the acquisition by decomposers of nutrients from inorganic pools; (5) death, or the accumulation of organic nutrients into organic nutrient pools; and (6) assimilation, or the acquisition of inorganic nutrients by autotrophs. The common basic structure assesses redundancy and functioning in ecosystems quantitatively and qualitatively. Each species within an ecosystem is represented by a population and each population has a finite probability of local extinction. Agriculture has provided increasing amounts of food for thousands of years despite frequent annual perturbations through cultivation, monocultures, and site-specific impacts (climate, toxins, etc.). As every ecosystem fails also does an agricultural one when it ceases to provide the services and goods demanded of it. Since Darwin, it is well known that perturbations lead to the extinction of populations, but it is also well known that agricultural production systems could be stabilized over thousands of years by increasing yields. What are the reasons for that? Could some varieties (key varieties) have overtaken tasks of extinct populations? Or does it mean that populations differ from each other by *resistance* strategies?

Biota respond to disturbances by a combination of *resistance* (the capacity to counteract threats) and *resilience* strategies (the capacity to recover). Resilience or recovering is a dynamic process and follows clear patterns of colonization and succession (probabilistic patchiness). It is governed by the availability of adaption strategies and refugia. In the face of external stress, sustainable units can maintain their structure (organization) and function (vigor) over time. On an ecosystem level, resilience is the display of positive adaption in the face of adversity and a comprehensive, multiscale, dynamic, hierarchical measure is *sustainability*.

Redundancy does not necessarily guarantee resiliency (sustainability) of ecosystem processes. Variation in species' richness within an ecosystem can mean variation in the number of functional

groups and variation in the number of species within and among functional groups. Obviously, the local extinction of species within functional groups is followed by compensatory growth of others, which generally leads to an effective replacement of the contribution of lost species to the overall group functioning.⁵ The compensatory abilities of species within functional groups seemingly stem from sustainability, meaning that local extinction of species within functional groups are inevitable and frequent. Reservoirs of species from adjacent ecosystems normally ensure that functional group or ecosystem failure, if it occurs, is likely to be transient. Increasing rates of global extinctions by increasing habitat fragmentation could mean that recolonization might play a decreasing role. Empirical demonstrations of dynamic stability and resilience are difficult and thus it is uncertain that species-rich systems are dynamically more stable, though it is well demonstrated that a greater biodiversity (plasticity) together with resistance and resilience strategies channel into more sustainability of agriculturally usable land.^{4,5} This is what is understood by “soil health.” Soil health is a continued capacity to function as a vital living system within natural or managed ecosystem boundaries by sustaining plant and animal productivity as well as maintaining, supporting, and enhancing air quality, water environments, and human health and habitation.

Biodiversity activity of agricultural production systems occurs in microaggregates, macroaggregates, rhizospheres (rhizoplane, ectorrhizosphere, endorrhizosphere), the detritosphere, the porosphere (region of water films channels between soil aggregates), macropores built up by root growth or micro- and macrofaunal tunnels (e.g., the drilosphere), the spermosphere, or phyllosphere. The most studied biological indicators are microbial biomass, basal- and substrate-induced respiration, mineralizable nitrogen, enzyme activities (e.g., dehydrogenases, phosphatases, sulphatases, ureases), abundance of microflora (bacteria, fungi, actinomycetes, algae), abundance of soil fauna (micro-, meso-, macrofauna), root disease, soil biodiversity (structural and functional biodiversity), food web structure, plant growth, and biodiversity (lower and higher plants). Soil microbial diversity is determined by lipid analysis, substrate utilization profiles, enzyme assays, and various nucleic acid analyses. Many conceptual and practical difficulties with the selection and use of biological indicators are left to describe soil health properly. Among others, just to mention the most important ones, are (1) the absence of any clear baseline data that might act as a reference point; (2) the identification of the most suitable bioindicators; (3) the assessment of health in view of the multitude of components that contribute to it; (4) the evaluation of systems that show no consistency in their responses to perturbations; (5) the high levels of spatial and temporal heterogeneity that affect all measurements in most systems; and (6) the lack of validation of bioindicators in diverse situations.

Currently, biodiversity is evaluated at three fundamental levels of biological organization: (1) on the *genetic diversity* level (variation in the components of nucleic acids), (2) on the *species biodiversity* level, and (3) on the *ecosystem diversity* level. Agricultural biodiversity is supposed to include all levels of abiotic ecosystem diversity and biological complexity, ranging from subspecies diversity (ecotypes, life cycles, genes, physiology, behavior) to species richness and supraspecies diversity (food web interactions, nontrophic relationships, above- and below-ground relationships).

Biodiversity in Agricultural Production Systems tries to cover most of the above aspects. Chapter 1 and chapter 2 cover the various facets of agricultural crops and cultivation practices. Chapter 3 gives an overview of the pore space dynamic in agroecosystems, where most of the soil microorganisms reside (bacteria, chapters 5–7; fungi, chapters 8–10; protozoa, chapter 11; nematodes, chapters 12 and 18; Tardigrada, chapter 13) or will live after introduction by soil amendments (chapter 4). The diversity of a larger-sized group of soil inhabitants, the earthworms and ants, is covered in chapter 14. The enzymatic diversity in soils is dealt within chapter 15. The many metabolic strategies available in soils ending finally as gaseous compounds (partially as greenhouse gases) and water are the subject of chapter 16 and chapter 17. The principles behind order and sustainability in natural successions and agriculture are covered in chapter 18. Chapter 19 gives an idea of how food chains in agricultural production systems can be modeled. Chapter 20, finally, discusses investigations of long-term observation plots to give an answer on how soil quality should be assessed.

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*Caminante, son tus huellas
el camino, y nada más:
caminante, no hay camino
se hace camino al andar.*

*Wanderer, es gibt keinen Weg,
Der Weg entsteht beim Gehen.*

*A road does not exist per se,
A road emerges during walking.*

Antonio Machado
(*Proverbios y cantares*, XXIX, 1917)

1 Diversity in Crop Production Systems

Bernd Honermeier

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1.1 DIVERSITY OF CROP PLANTS

1.1.1 CEREALS AND PSEUDOCEREALS

In comparison with plant species distributed in the wild flora, the diversity of agricultural crops is strongly limited. Only a minor number of plant species are used for the production of foods, feedstuffs, and industrial products. That is particularly applying to the cereal species, which have the greatest cultivation area of all agricultural crops worldwide. In 2004 around 76% of the total world cereal area were cultivated only with three main cereal species: wheat (*Triticum aestivum*) with a relative part of 32%, rice (*Oryza sativa*) with a relative part of 23%, and maize (*Zea mays* L.) with a relative part of 21%. The rest of this area are particularly distributed the cereal species barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), sorghum (*Sorghum bicolor* L.), millet (*Pennisetum americanum*, *Setaria italica*, *Panicum miliaceum* and other), durum wheat (*Triticum durum* L.), oats (*Avena sativa* L.), and triticale (*Triticosecale* Wittm.). During the last years a slight reduction of the growing area of barley, rye, and oats was observed, which shows that in the future the crop diversity of cereals will continue to reduce and focus on the main cereal crops wheat, rice, and maize (table 1.1).

To increase the crop diversity on arable land, a number of plant species are known, which can be cultivated in Europe and in other regions. In cereals there is spelt wheat, (*Triticum spelta* L.) with a good cultivation ability in most regions. Presently spelt is cultivated in a small area in Middle Europe (South Germany, Switzerland, Austria, Belgium). Besides spelt other wheat species are

TABLE 1.1
Cultivation Area of Cereals in the World from 1970 to 2004¹⁷

Cereal crops	1970	1980	1990	2004
Cereals world (1000 ha)	675.691	717.494	708.459	681.237
(relative)	(100)	(100)	(100)	(100)
wheat	31	33	33	32
rice (paddy)	20	20	21	23
barley	10	11	10	8
maize	17	17	19	21
rye	3	2	2	1
oats	4	3	3	2
millet	7	5	5	5
sorghum	7	6	6	7

TABLE 1.2
Old Wheat Species (*Triticum* sp.) with Descriptions of Ear Characteristics⁴

Wheat species	Scientific name	Ear characteristics
Small spelt	<i>Triticum monococcum</i>	fragile ear axis, strong hulls
Emmer	<i>T. dicoccum</i>	fragile ear axis, strong hulls
Rivet	<i>T. turgidum</i>	tough ear axis, loose hulls
Polish wheat	<i>T. polonicum</i>	tough ear axis, loose hulls
Spelt	<i>T. aestivum</i> , ssp. <i>spelta</i>	fragile ear axis, strong hulls
Common wheat, ssp. <i>compactum</i>	<i>T. aestivum</i> , ssp. <i>compactum</i>	tough ear axis, loose hulls
Dwarf wheat	<i>T. aestivum</i> , ssp. <i>sphaerococcum</i>	tough ear axis, loose hulls

known that were partially involved in the evolution of common wheat (*Triticum aestivum* L.) by hybridization of several *Triticum* species (table 1.2). These wheat species are not taken into account in breeding programs. That’s why they are characterized by a low grain yield potential, which is only around 40 to 60% of the grain yield of common wheat (*Triticum aestivum* L.) that is primary cultivated in the world. These low grain yields are due to the reduced number of seeds per ear, reduced stem stability, and the reduced disease resistance. Old wheat species can not use the nutrients and other growing factors with the same efficiency like common wheat.

Other species from the family of *Poaceae* that can be used and cultivated as starch-containing grain crops are millets like *Eragrostis tef* and *Eleusine coracan* ssp. *africana*, couch-grass (*Thiopyrum intermedium* L.), and water rice (*Zizania palustris* L.). Also pseudo-cereals, which are not a member of *Poaceae*, could contribute to the increase of crop diversity. This includes buckwheat (*Fagopyrum esculentum* Moench), grain amarant (*Amaranthus* spp.), and quinoa (*Chenopodium quinoa* L.). Buckwheat is cultivated as a grain crop in Central Asia (China), East Asia (Japan), and East Europe. The leaves of buckwheat plants contain the flavonoid quercetin-3-O-rutinosid with a content of 2–6% of leaf dry matter. This flavonoid has positive pharmacological effects. That’s why buckwheat is used as a medicinal plant. Grain amarant and quinoa are cultivated in a small area in Latin and South America. All pseudo-cereals can be used in dietetics in therapy of gluten allergy diseases because of the absence of gliaden and glutenin proteins in their seeds.

1.1.2 OIL SEED CROPS

A reduced crop diversity exists also in the cultivation of oil seed crops. Looking at the results of the year 2004 the total world growing area of oil seed crops was about 247 Mio. ha.¹⁷ From that are cultivated a relative part of 36% with soybean, 14% with cotton (seed using), 11% with ground nut, 10% with rape seed, and 10% with sunflower. Increasing the crop diversity of oil crops can be reached by including new and underutilized species. During the last few years field experiments have shown that several oil crops with specific fatty acid composition can be cultivated in many regions. Most of them are summer oil seed crops like false flax (*Camelina sativa* L.), white mustard (*Sinapis alba* L.), black mustard (*Brassica nigra* L.), crambe (*Crambe abyssinica* L.), safflower (*Carthamus tinctorius* L.), poppy (*Papaver somniferum* L.), and oil squash (*Curcubita pepo* L.). Some of them are characterized by positive properties like less water and nutrient demand, short plant development, suitability for growing under low input conditions, and specific seed and oil quality. A new oilseed crop for the future could be the wild plant cuphea (*Cuphea viscosissima* Jacq. × *C. lanceolata* W. T. Aiton) to substitute small- and medium-chain triglycerides, which are in high demand for chemical manufacturing.²¹ Domesticated genotypes of cuphea show a good potential for agricultural production, but their indeterminate growth may result in seed shatter during the ripening phase.²¹ Other primary barriers to commercial production of *Cuphea* spp. have been seed dormancy and self-incompatibility.²²

1.1.3 PULSES, ROOT CROPS, AND TUBER CROPS

In 2004 the world pulses area (without soybean) was around 72 Mio. ha. Inside this area the grain legumes occupied a relative part of 38% dry beans (*Phaseolus vulgaris* L.), 15% chick peas (*Cicer arietinum* L.), and 14% cow peas (*Vigna sinensis* L., *V. unguiculata* L.). The rest of the pulses area was planted particularly with dry peas (*Pisum sativum* L.) (9%), pigeon peas (*Vicia faba minor* L.) (6%), lentils (*Lens culinaris* L.) (5%), and broad beans (*Vicia faba major* L.) (4%).¹⁷ Several more legumes are cultivated as grain or tuber crops in tropical and subtropical regions. Some of them are yam bean (*Pachyrhizus erosus* L., *P. ahipa* (Wedd.) Parodi, *P. tuberosus* (Lam.) Spreng.), bambara groundnut (*Vigna subterranea* L.), grass pea (*Lathyrus sativus* L.), sword bean (*Canavalia gladiata* (Jacq.) DC., *C. ensiformis* L.), catjang (*Cajanus indicus* L.), cluster bean (*Cyamopsis psoralioides* (Lam.)), and goa bean (*Psophocarpus tetragonolobus* (Stickm.)). These crops can be characterized as underutilized crops, because they are only locally used for food.

In 2004 the total area of root and tuber crops was about 53 Mio. ha.¹⁷ The greatest parts of that were cultivated with potatoes (*Solanum tuberosum* L.) (36%), cassava (*Manihot esculenta* Crantz) (34%), and sweet potatoes (17%) (*Ipomoea batatas* L.). Further root crops that are locally used in tropical regions as edible crops are yam (*Dioscorea* sp. L.), taro (*Colocasia esculenta* L.), arrowroot (*Maranta arundinacea* L.), and tannia or cocoyam (*Xanthosoma sagittifolium* L.). All these crops particularly are accumulating carbohydrates (starch, glucose, saccharose) in their tubers or roots. They are used for human nutrition and partially for technical application.

1.1.4 SUGAR PLANTS

For sugar production only two crops are dominating worldwide—sugar cane (*Saccharum officinarum* L.) and sugar beets (*Beta vulgaris* L.). Both crops accumulate the disaccharide saccharose in the roots (sugar beets) or in the internodes of the stems (sugar cane). An increased biodiversity of crops for producing sugar is possible. A good ability for cultivation is known for chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.). Root chicory is cultivated particularly in Belgium, France, and South Africa with around 25.000 ha.¹⁷ The cultivation

area of the Jerusalem artichoke is very small. Both crops belong to the family of *Asteraceae*. They are able to synthesize fructan (inulin) in the roots or in the tubers with a relative inulin content of around 15 to 18%. An alternative sugar crop is *Stevia rebaudiana* (Bertoni), cultivated and used in America and in East Asia (Japan). The sweetening agents of this plant are diterpen glycosides with steviosid as the main component located in the leaves. First field experiments have shown that *Stevia rebaudiana* can be cultivated also in Middle Europe.⁴⁰ A further underutilized crop is common licorice (*Glycyrrhiza glabra* L.), which contains Glycyrrhizin in the roots. This plant is used traditionally to produce licorice or licorice-sticks. Besides this *Glycyrrhiza glabra* is also used as a medicinal plant because of their expectorant and antiphlogistic effects.

1.1.5 DIVERSITY OF MEDICINAL AND SPICE PLANTS

An increased crop diversity exists in medicinal, spice, and dye plants. But only a minor part of these plants are cultivated. Most of them grow in the wild flora and are collected manually for use. A conversion from manual collecting to established growing techniques is necessary to improve drug yields and reach good drug quality.

Production technology, especially harvesting technology for medicinal and spice plants, is dependent on the product harvested, which can be leaves, respectively herbs, flowers, seeds, or roots. Occasionally resins, bark, or the whole plant are used. Therefore, the most important species of medicinal plants are listed in table 1.3 with regard to the parts of the plants used. In addition to the commercial use of medicinal plants, a high number of spice plants and dye plants is known and cultivated in private gardens.

However, cultivation of medicinal and spice plants is not without risks and especially cultivation of new cultivars requires a certain amount of experience.²⁵ Many medicinal plants still show the characteristics of wild plants: they do not germinate well, do not mature evenly, or do not burst

TABLE 1.3
Important Medicinal Plants Used as Leaf, Herb, Flower, Seed, and Root Drugs ²⁵

Plant species	English name	Family	Ingredients
<i>Matricaria recutita</i>	Chamomile	<i>Asteraceae</i>	Essential oil (bisabolol, chamazulen), flavonoids
<i>Arnica montana</i>	Arnica	<i>Asteraceae</i>	Essential oil, flavonoids
<i>Calendula officinalis</i>	Marigold	<i>Asteraceae</i>	Flavonoids, saponines, caratinoids, essential oil
<i>Lavandula angustifolia</i>	Lavender	<i>Lamiaceae</i>	Essential oil (linalool, linalylacetat)
<i>Carum carvi</i>	Caraway	<i>Apiaceae</i>	Essential oil (carvon)
<i>Foeniculum vulgare</i>	Fennel	<i>Apiaceae</i>	Essential oil (trans-anethol, fenchon, methylchavicol)
<i>Silybum marianum</i>	Milk thistle	<i>Asteraceae</i>	Flavonoids (silybin, silydianin, silychristin)
<i>Oenothera biennis</i>	Evening primrose	<i>Onagraceae</i>	Fatty oil with γ -linolenic acid
<i>Borago officinalis</i>	Borage	<i>Boraginaceae</i>	Fatty oil with γ -linolenic acid
<i>Papaver somniferum</i>	Poppy	<i>Papaveraceae</i>	Alkaloids (morphin)
<i>Valeriana officinalis</i>	Valerian	<i>Valerianaceae</i>	Valeopotriates, essential oil
<i>Gentiana lutea</i>	Yellow gentian	<i>Gentianaceae</i>	Bitter substances
<i>Inula helenium</i>	Elecampane	<i>Asteraceae</i>	Bitter substances, essential oil, inulin
<i>Angelica archangelica</i>	Angelica	<i>Apiaceae</i>	Cumarines, essential oil
<i>Armoracia rusticana</i>	Horse radish	<i>Brassicaceae</i>	Mustard oil
<i>Echinacea purpurea</i>	Purple Coneflower	<i>Asteraceae</i>	Echinacosid, polysaccharides, essential oil

easily. In order to harvest root, herb, and flower drugs, very often special harvesters are needed, which have to be specially constructed for this purpose or be bought. The planting of the crop is equally problematic; not all varieties can be sown directly onto the field, but have to be raised in nursery beds and then transplanted. This again affords the necessary technical equipment (greenhouses, planting machines) or investments in the purchase of seeds and wage costs for manual work. Weed control represents a further major problem. According to the new European herbicide regulations, which grants authorization for indicated uses of herbicides only, herbicides can exclusively be used for those plants they have been tested and given authorization for. Generally, this does not apply to medicinal plants. Therefore, at present groups of pharmaceutical companies, cooperatives, and state institutions try to attain supplementary regulations for the use of herbicides on medicinal plants. Only after the regulations in question have been passed will herbicides be allowed to be implemented in this area. Otherwise, weed control has to be conducted mechanically, and very frequently even manually. In crops with slow and uneven germination and lack of ground cover, this can be a factor for an increase in costs and working time.

The effective ingredients of medicinal plants, like those of spice plants and dye plants, are mostly secondary metabolic products. In contrast to primary metabolic products, like carbohydrates, proteins, and fat, only small amounts of these products can be found in the plants, and they are normally characteristic of certain groups of plants. They often are found only in certain cells or cell groups and sometimes only during certain phases of differentiation. The content of active substances within a plant can vary considerably, depending on the genotype and environment (climate, soil, season, and time of day). Pharmaceutical terminology refers to the chopped-up and dried vegetable material as drug. Since the drug is the basic material for further industrial processing, it should contain all the active substances relevant to the pharmaceutical product.

Basic materials of phytopharmaceuticals can be categorized in different ways, depending on their various properties. They can be categorized from a pharmaceutical point of view according to their effectiveness, or be categorized according to special characteristics such as color, taste, smell, solubility, basicity, and effectiveness. Group designations for pharmacologically effective content materials, which are still applied nowadays, have been the result of these categorizations.²⁵

- essential oils
- alkaloids
- flavonoids
- cardio-effective glycosides
- saponines
- bitter substances
- slimy substances
- tannic acids
- anthraglycosides

The chemical diversity of medicinal plants is very high. In many investigations a wide range in the content of ingredients within the species was observed. That was also found in Hawthorn (*Crataegus* sp.), Caraway (*Carum carvi*), Fennel (*Foeniculum vulgare*), Nasturtium (*Tropaeolum majus* L.), and in many other plants (table 1.4). The reason for that are the genetics of the plant as well as the morphological and physiological characteristics. Contents and distribution of secondary metabolites within the plants are also dependant on growing conditions.

The variability of secondary metabolites within and between the species can be shown with phenolic compounds, which can be found in many plant species. Factors determining the diversity of phenolic acids in medicinal plants, fruits, and vegetables are the genetics, morphology, physiological stage, environmental conditions, agronomy, and processing of the plants. Within the species many chemotypes have been found or genotypes have been selected. The concentrations of phenolic acids like chlorogen acid, hydroxycinnamic acid, or hydroxybenzoic acid are generally highest in

TABLE 1.4
Chemical Diversity of Selected Medicinal Plants

Medicinal plant	Minimum	Maximum
Hawthorn (<i>Crataegus</i> sp.) ³⁷		
flavonoides	0.28%	1.92%
procyanidines	0.38%	1.94%
Caraway (<i>Carum carvi</i>) ³⁵		
essential oil	0.54%	6.26%
Carvon	20.8%	80.1%
Fennel (<i>Foeniculum vulgare</i>) ³⁴		
essential oil	2.2%	12.1%
Anethole	21.2%	61.8%
Fenchone	22.3%	53.0%
Estragole	0.39%	2.19%
Nasturtium (<i>Tropaeolum majus</i> L.) ²⁸		
Glucotropaeoline		
flowers	13%	31 mg/g TM
leaves	14%	29 mg/g TM

young leaves or fruits and decrease during plant development. External factors effecting phenolic acids are light, temperature, and various abiotic and biotic stresses. Phenolic acids are directly implied in the response of plants to mechanical, chemical, or microbiological stress. Compounds that are involved are already present in the plant or are formed after injury or biosynthesized de novo. Agronomic factors affecting the concentration of phenolic compounds are nitrogen fertilization and irrigation. It was found that both factors lead to a reduction of flavonoids or other phenolic compounds in medicinal plants.

1.2 EFFECT OF PLANT CULTIVATION ON BIODIVERSITY

1.2.1 MANAGEMENT OF CROP ROTATIONS

Contrary to the cultivated crops with homogeneous and mainly annual or biannual plant populations, we find in wild flora mainly perennial plant populations with high diversity. This disadvantage in crop cultivation has to be compensated by systematic rotation of crops at the cultivated fields. For that reason crop rotations are a very important tool in crop management on arable land, which can influence the yields as well as the quality of the harvested products of the cultivated plants. The term “crop rotation” is defined as a systematic rotation of crops in the same field and at a limited length in time. The alternation of crops takes place from year to year. Within one year it is to differ between major crops and intermediate crops. Major crops like wheat, maize, sugar beets, soybeans, or potatoes are full-season crops. They are very important for the income of the farmers. Intermediate crops like white mustard, turnip rape, buckwheat, or fodder radish have a short plant development. They are cultivated only for some weeks between two major crops. The main targets for cultivation of intermediate crops are the improvement of the soil fertility, the reduction of nematode populations in the soil, and the production of forage.

Depending on the region there exists a wide range of different crop rotations. The type and the diversity of crop rotations depends on soil and climate conditions as well as on farming systems, growing techniques, and economics. For economical reasons profitable cash crops like wheat, rapeseed, sugar beets, soybeans, or maize comprise the greatest part within crop rotations in most

TABLE 1.5
Examples of Crop Rotations Used in North America and in Germany ^{23,26,51}

North America (USA, Canada)	Germany
Soybean–wheat	Sugar beet–wheat–wheat
Soybean–corn	Rape seed–wheat–wheat–barley
Fallow–sunflower–wheat	Rape seed–wheat–barley
Lens–wheat	Corn–wheat
Fallow–turnip rape–wheat	Potato–rye–corn–corn
Fallow–wheat	Peas–rape seed–wheat–rye
Fallow–sunflower–wheat	Field beans–wheat–barley–oats
Fallow–wheat–corn–sorghum	Red clover–red clover–potato–wheat–field beans–wheat–rye
Turnip rape–wheat	Corn–field beans–wheat–barley
Rape seed–wheat	Potato–triticale–rye

regions. On fertile soils only two or three crops are of widespread use within one crop rotation. Some examples for representative crop rotations in North America and Middle Europe with little and high numbers of crop rotation links are shown in table 1.5.

Crop rotations with increased crop diversity are used especially in organic farming. Due to the fact that synthetic pesticides and synthetic mineral fertilizers are not allowed in this farming system it is necessary to use the natural regulation functions of a crop rotation. An important factor of crop rotations in organic farming is the establishment of legumes, which are fixing air nitrogen. This nitrogen can be accumulated in the soil and is available for the following crops. Typical legume crops that are cultivated in agricultural systems in Europe are red clover (*Trifolium pratense* L.), alfalfa (*Medicago sativa* L.), field beans (*Vicia faba* L.), and peases (*Pisum sativum* L.). In most cases crop rotations in organic farming contain more than five fields. Typical crop rotations in organic farming in Europe are:

1. Red clover–red clover–potato–wheat–field beans–wheat–rye, or
2. Corn–field beans–wheat–barley–clover/grass–clover/grass

To establish new crop rotations specific rules have to be considered. The most important factors are soil properties like soil type, water capacity, and soil structure as well as plant characteristics like plant development (sowing and harvest time), resistance against diseases, nutrition demand, competition against weeds, and allelopathic interactions between plants. An important factor for managing crop rotations is the probability for infection with soil-born diseases (fungi and virus diseases) and pests, which depend on the relative part of potential host plants in the crop rotation. Some examples for the interaction between crop rotation and disease risk are given in table 1.6. To reduce the disease risk a suitable sequence of crops has to be considered and a maximal concentration of crops within the rotation has to not be exceeded.

The diversification of cropping systems lead to many effects on the soil characteristics (physical, chemical, microbiological characteristics), on nutrient dynamics of the soil, on plant growth, and on the environment. These effects could be observed in several field experiments executed under different climate and soil conditions.²² The effects of diversification of crop rotations are modified by the cropping system (tillage, fertilization) as well as the local soil and climate conditions. They cannot be generalized for all conditions and regions.

In recent years changes in diversification and intensity of cropping systems in the Great Plains of North America have been observed. For this region the effects of diversification have been investigated. They are summarized in table 1.7.

TABLE 1.6
Disease Risk Associated with Various Crop Rotations in the Northern Great Plains/U.S.²⁶

Crop rotation	More risk of	Less risk of
Canola–lentil/pea–wheat	<i>Ascochyta</i> sp. <i>Sclerotinia</i> sp. Blackleg	Most diseases
Canola/sunflower–wheat–pea–wheat	<i>Sclerotinia</i> Leaf spots <i>Fusarium</i> sp.	None
Canola–barley–barley–flax–wheat–wheat	Leaf spots <i>Fusarium</i> sp. Common root rot Take-all	Blackleg <i>Sclerotinia</i> sp.
Pea/flax–wheat–canola/flax–oat/barley	<i>Fusarium</i> sp. Flax wilt <i>Sclerotinia</i> sp.	<i>Ascochyta</i> sp. Leaf spots Root rots

TABLE 1.7
Effects of Diversification of Crop Rotations in the U.S. and Canada²³

- 1. Effects on the soil
 - Increasing the root residues
 - Increasing the content of carbon and organic matter
 - Improvement of the water capacity
 - Growth of mykorrhiza
- 2. Effects on plants
 - Improvement and stabilization of yields
 - Lower infection with soil-born pathogens
 - Reduction of weed populations
 - Reduction of populations with nematodes
 - Improvement of the nutrient uptake
 - Improvement of the root distribution
- 3. Ecological effects
 - Increasing the emission of N₂O
 - Reduction of pesticide application

The diversification of cropping systems can lead to higher crop yields by influencing plant diseases, weeds, root distribution, moisture utilization, and nutrient availability.²³ Diversified crop rotations can also alter the pattern and degree of nutrient removal. Increasing amounts of crop residues returning to the soil can increase the soil organic C pool and can lead to greater potential for the nutrient cycling, an effect that will increase with time.^{10,23} This effect on organic matter content and on nutrient recycling will increase by reducing the tillage system and by including legumes in crop rotations. The crop rotation may also have an important influence on nutrient uptake of crops. For instance, the phosphorus nutrition of crops depends on the preceding crop due to its effects on the activity of vesicular–arbuscular mycorrhizae (VAM). VAM fungi form symbiotic relationships with many plant species and can increase the zone of absorption of immobile phosphorus. It is reported that the type of preceding crop, the crop residues, and the

phosphorus fertilization can affect the VAM activity and therewith also the phosphorus uptake of the crops.^{11,47}

1.2.2 USE OF ALLELOPATHY

The phenomenon of allelopathy is defined as biochemical interaction between all types of plants, including microorganisms. Plants release chemical compounds into the environment through root exudation, leaching by dews and rains from the plant surfaces, decaying plant tissues, and by volatilization. Many chemical compounds are known and well documented with their allelopathic effects. Most of the allelopathic compounds of the plants can be characterized as secondary metabolites like phenolic acids, flavonoids, cyanogenic glycosides, tannins, isothiocyanates, and others. A total of 16 potential allelochemicals have been found in rice.³² Most of them are phenolic acids like p-salicyclic acid, p-coumaric acid, vanillic acid, syringic acid, ferulic acid, hydroxamic acid, and mandelic acid. All these have inhibitory activity. For example, p-coumaric acid inhibits the germination of lettuce (*Lactuca sativa* L.) seedlings.⁴² A further group of secondary metabolites with allelopathic potential are isothiocyanates (ITC) contained in *Brassica*-species. ITC released by turnip rape mulch (*Brassica rapa* L.) was investigated under field conditions in Germany.³⁸ The isothiocyanates were strong suppressants of germination on tested weed species—prickly sowthistle (*Sonchus asper* L.), scentless mayweed (*Matricaria inodora* L.), smooth pigweed (*Amaranthus hybridus* L.), barnyardgrass (*Echinochloa crusgalli* L.), blackgrass (*Alopecurus myosuroides* Huds.), and wheat (*Triticum aestivum* L.).³⁸ It can be concluded that this effect can be used for the following crop, especially under no tillage conditions to suppress weeds.

Further allelochemicals can be synthesized by fungal pathogens. Some fungal metabolites are involved in the inhibition and modification of plant growth and development. An example for that are the secalonic acids, a series of ergochrome pigments, exist in a group of food-born fungal metabolites.⁵⁰ These fungi produce several secalonic acids when they grow on cereals like rye (*Secale cereale* L.). It could be found that these fungal metabolites have a highly phytotoxic potential. The inhibition of plant growth of these fungal metabolites is due to the cell ultrastructure destruction and the reduction of photosynthesis and root activities.⁵⁰

Several crops have been identified that have allelopathic properties. Accessions with an allelopathic potential have been found in crops such as sugar beet (*Beta vulgaris* L.), yellow lupine (*Lupinus luteus* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), pea (*Pisum sativum* L.), and cucumber (*Cucumis sativus* L.).^{41,16} Also in germplasm of rice (*Oryza sativa* L.) several accessions and cultivars with allelopathic effects have been found in laboratory and under field conditions.^{16,31} Further crops with allelopathic potential are legumes like velvetbean (*Mucuna deeringiana* Bort.), jackbean (*Canavalia ensiformis* L.), and jumbiebean (*Leucaena leucocephala* (Lam.) de Wit). These legumes can reduce weed growth and improve the yields of corn during the following two years of cultivation.⁹

Among the crop cultivars are differences in allelopathic potential. That could be found in rice where a large variation in allelopathy among cultivars has been observed.³¹ An example for a highly allelopathic cultivar in rice is IAC 165, a *japonica* upland variety bred in Brazil. Laboratory screenings have shown that a large proportion of Brazilian upland rice germplasm is allelopathic, which is different from those of Asian rice germplasm.³¹ The knowledge about the variation of the allelopathic potential of the cultivars is important for crop management to use cultivars, which are suitable for weed control. The use of cultivars with an allelopathic effect on weeds can mean lower production costs because the need for herbicide application is reduced.

Generally it can be stated that the research on allelopathy is a young discipline in crop sciences. Until now the use of this phenomenon in agricultural practice is low. Many investigations are

necessary to identify allelopathic agents, to understand the mechanisms of allelopathy, and to identify genes encoding for allelopathy in crops like rice.

1.2.3 CROPPING SYSTEMS

The individual crop is effected by the cropping system of which the crop is a component. The ecological conditions under which the crop is grown are determined by many factors. Thus are the soil, the atmospheric environment, and cultivation methods including crop rotation.³⁰

Cropping systems are characterized by different cultivation methods (tillage or no tillage, with or without irrigation, bed or dam cultivation), by different intensity of cultivation (pesticide application, frequency and dose of mineral and organic fertilization), and by different cultivation frequency (duration of the cropping phase as a percentage of the total duration of the cultivation cycle). That means a wide range of cropping systems exists depending on the local conditions. Tropical cropping systems can be classified according to Norman et al. (1995):

- Shifting cultivation systems
- Semi-intensive rainfed systems
- Intensive rainfed systems
- Irrigated and flooded systems
- Mixed annual/perennial systems

The principals and characteristics of the above-mentioned cropping systems are described from Aufhammer (1999) and Norman et al. (1995).

In most regions of arable land worldwide sole cropping systems are executed. These are characterized by only one crop and only one cultivar cultivated in the same field during the same vegetation period. We find, for example, sole cropping in the cultivation of wheat, barley, rye, rapeseed, linseed, corn, sugar beet, soybean, pea, sorghum, or sugar cane. For that reason within one field the diversity of crops is very low. On the other hand, an increased number of cultivars, which are officially licensed by the government, was observed the two decades in Europe. For example, in Germany the number of licensed cultivars increased from 1980 to 2004 in winter wheat from 46 to 111, in winter barley from 35 to 83, in triticale from zero to 29, in winter rape from 27 to 58, in potatoes from 130 to 205, and in corn from 47 to more than 150 cultivars.⁸ It can be concluded that contrary to the individual fields with low crop diversity within a whole region of arable land in Germany the diversity of cultivars has been increased during the last two decades.

Besides this conventional cropping system mixed cropping can be used. Mixed or intercropping can be divided in full intercropping (simultaneous cultivation of several crops or cultivars in the same field) and in relay intercropping (temporary mixed cropping, for example, underseed of clover under cereals). Some examples of intercropping systems are given in table 1.8.

Intercropping systems can also be classified into different methods regarding the distribution of the crops in the field. The most important methods of intercropping are described in figure 1.1.

The aims of these intercropping methods can be described as follows:

- Improved use of the natural resources (water, nutrients, light impact, vegetation period),
- improvement of yield stability and resistance to lodging,
- reduction of abiotic and biotic stress factors, and
- improvement in the nutrient uptake of the crops.

Furthermore, we can classify the terms *sequential cropping*, *monoculture*, and *crop rotation*. Sequential cropping is characterized by a sequence of two or more crops in the same field in the same vegetation period. One example for that is the combination of a main crop like wheat and a catch crop like turnip rape during the same vegetation period. Monoculture can be characterized

TABLE 1.8
Intercropping Systems with Different Crop Species
(selected examples from temperate climate regions)

Species	Targets
Forage crops	
Red clover + rye grass	Forage yield, biological nitrogen fixation
Grains	
Maize + soybean ²⁷	Silage production
Oats + wheat ⁵	Forage yield
Barley + rye grass ⁴⁶	Forage yield
Rye + hairy vetch ¹²	Mulching, soil conservation, nitrogen accumulation
Oats + alfalfa ⁴⁸	Reduction of erosion at slopes
Bean + peas ⁴⁴	Reduction of lodging
Lupine + maize ²	Reduction of water leakage
Wheat + triticale ⁴³	Ethanol production

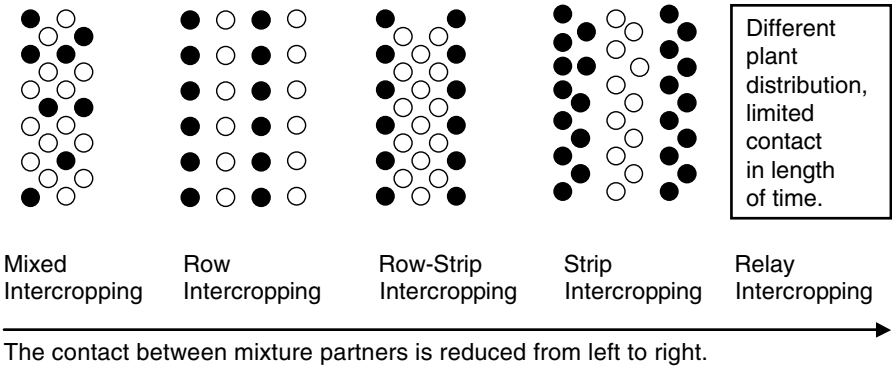


FIGURE 1.1 Methods of intercropping³

as a sequence of the same crop over several years. Examples for that we find in the cropping of maize and wheat, which are cultivated in some regions in monoculture.

1.2.4 SOIL TILLAGE

Soil tillage systems can be classified into conventional tillage (reversible tillage with plough); reduced tillage, also called conservation-tillage or minimum-tillage; and no-tillage (direct drilling without tillage). Conventional tillage is taken with a plough, which turns over the soil in a depth to around 25 to 30 cm. This tillage system leads to an intensive mixing of the soil with organic matter and organic residues from the field (roots, straw, stubble, manure). Furthermore, conventional tillage leads to an effective mechanical weed control as well as to an intensive loosening of the soil. The last mentioned effect induces an increased mineralization of organic matter leading to lower content of organic matter as well as to increased content of mineral-available nitrogen (nitrate). Another important effect is that conventional tillage systems tend to increase erosion. After ploughing the soil surface is not covered with plant residues and for that reason the soil is not protected against erosion. Mulching in combination with conservation tillage can be an alternative cultivation method to reduce this effect. For that reason conservation tillage was introduced in farming systems in North America around 40 years ago. In recent years minimum tillage systems became more and more widespread also in European countries.

TABLE 1.9
Influence of Different Tillage Methods on Earthworm Activity at the Experimental Station Angerstein, Spring 1993³⁶

Parameters of earthworm activity	Tillage		
	Conventional ^a	Reduced ^b	Without
Number of earthworm/m ²	19	40	117
Part of adults (%)	36	45	22
Biomass (g/m ²)	43	111	160
Diversity index H ₅	0.20	0.37	0.53
^a yearly ploughing			
^b one year of four ploughing			

Reduced tillage can change numerous physical, chemical, and biological properties of the soil. A number of field studies have been conducted to determine the effects of varying tillage practices on soil fertility.^{49,6,29,36,24,19} In some investigations an increased number of earthworm populations could be observed in reduced tillage systems (table 1.9).

Contrary to ploughing in minimum-tillage systems, only a small number of earthworms and earthworm canals in the soil will be destroyed. Furthermore, reduced tillage systems lead to more plant residues covering and protecting the soil surface. For that reason the soils in which a reduced tillage is taken have better conditions to reproduce the earthworm populations. Additional effects of reduced tillage are changes in nutrient content (potassium, nitrogen) in the soil. Long-term no-till management has resulted in increased nitrogen content in the soil and in pronounced vertical stratification of soil exchangeable potassium.⁴⁹ Significantly higher soil K concentrations in the surface layer and lower K levels at subsurface depths have been observed in comparison with moldboard plow. This vertical soil K stratification is mainly attributed to limited soil mixing, surface application of K fertilizer, deposition of crop residue at the soil surface, and the relative immobility of K in soil.⁴⁹ Furthermore, more efficient moisture use and improved soil properties associated with no-tillage have been documented (table 1.10). No-tillage and reduced tillage systems are an effective step in efficiently saving more precipitation for crop production.

Reduced tillage systems have also been shown to increase soil mechanical resistance, which can delay root development and increase water stress if dry conditions occur early in the growing season.⁶

TABLE 1.10
General Effects of Reduced Tillage Systems^{24,19}

Property	Tillage	
	Conventional	Reduced
Earthworm population	+	–
Erosion	–	+
Soil structure, soil fertility	+	–
Infiltration of rain water in the soil	+	–
Evaporation	+	–
Content of organic matter in the soil	–	+
Infection with fungi pathogens (<i>Fusarium</i> sp., <i>Pseudocercospora</i> <i>herpotrichoides</i> , <i>Gaeumannomyces</i> <i>graminis</i> , <i>Helminthosporium</i> <i>tritici repens</i>)	+	–
Weed density	–	+

No-tillage induces lower soil temperatures, which result in slightly lower (1 – 2d) seedling emergence rates, which has been observed in experiments with maize. Lower soil temperature could be a result of reduced air flow in the soil in conservation tillage. A further important effect of tillage is focused on weed population and weed density. No-tillage and reduced tillage systems lead to an increased number of short-cycle species (most monocotyledon species) as well as on long-cycle species (most dicotyledon species) of the weed population. In Europe increased populations of the weeds *Alopecurus myosuroides* Huds., *Bromus* L. spp., *Chenopodium album* L., and *Stellaria media* L. have been observed in field experiments with reduced tillage systems (table 1.11). Generally it can be concluded that reduced tillage systems require higher frequency of herbicide application.

TABLE 1.11
Influence of Different Methods of Tillage on Seed Bank and
Number of Emerged Seedlings m² of *Chenopodium album* L.,
Experimental Station Gross Enzersdorf²⁴

Seed bank in the soil	Tillage		
	Conventional	Reduced	Zero
Seedlings m ²	12	522	4043
Seeds m ²			
in 0–5 cm	6814	62972	205477
in 5–10 cm	9230	20185	12249
in 10–20 cm	23550	21652	89109
in 20–30 cm	27000	18805	15352

A further negative effect of reduced tillage systems is focused on the infection of plant diseases like *Fusarium* sp., *Pseudocercospora herpotrichoides*, *Gaeumannomyces graminis*, and *Helmintosporium tritici repens*, which are important pathogens in cereal cultivation (table 1.10). These necrophytic fungi pathogens survive on plant residues covering the soil surface at the field. In reduced tillage and no-tillage systems these plant residues, which are infected with fungi pathogens, are not incorporated in the soil. For that reason the pathogens have favorable conditions for their development and reproduction. In spite of the above-mentioned negative effects, the reduced tillage systems are widespread and well accepted in crop cultivation. That is also due to the increased interest in reduced tillage and no-tillage systems, which has resulted from a need to conserve energy, reduce soil erosion, and improve profitability of the farms.

1.2.5 CULTIVATION OF GENETICALLY MODIFIED PLANTS

Genetically modified plants (GMPs) were first released commercially in 1992. Their global area covered around 10 million hectares beginning in the 1990s and around 30 million hectares at the end of the 1990s. In 2003 approximately 56 million hectares of GMPs were grown in the world.⁴⁵ The first genetically modified crop introduced in practice cultivation has been the oil plant soybean, in which a herbicide tolerance was established. A short time later it was also established in rapeseed (*Brassica napus* L.), maize (*Zea mays* L.), sugar beet (*Beta vulgaris* L.), and potato (*Solanum tuberosum* L.), which are together with soybean the five main genetically modified crops established with the traits of herbicide tolerance and/or insect resistance. Presently some other genetic traits like modified fatty acid composition in the seed oil of rapeseed and soybean or induced male sterility to produce hybrids in maize as well as insect resistance (potato, maize, cotton) and delayed ripeness (tomato, carnation) are utilized (table 1.12).

In 1998 the first commercialized GMP was cultivated in the European Union with insect-resistant maize in Spain and France. Other crops being developed for commercial application in

TABLE 1.12
List of Approved Products of Genetically Modified Plants in the World¹

Crop	Species	Number of products	Genetic traits
Argentine Canola	<i>Brassica napus</i> L.	15	HR, MFAC, MS
Sugar Beet	<i>Beta vulgaris</i> L.	3	HR
Polish Canola	<i>Brassica rapa</i> L.	2	HR
Creeping Bentgrass	<i>Agrostis stolonifera</i> L.	1	HR
Papaya tree	<i>Carica papaya</i> L.	1	VR
Chicory	<i>Cichorium intybus</i> L.	1	MS
Melon	<i>Cucumis melo</i> L.	2	MM, DR
Squash	<i>Curcubita pepo</i> L.	2	VR
Carnation	<i>Dianthus caryophyllus</i> L.	3	MC, HR, DR
Soybean	<i>Glycine max</i> L.	7	HR, MFAC
Cotton	<i>Gossypium hirsutum</i> L.	9	HR, IR, MM
Sunflower	<i>Helianthus annuus</i> L.	1	HR
Lentils	<i>Lens culinaris</i> Medik.	1	HR
Linseed	<i>Linum usitatissimum</i> L.	1	HR
Tomato	<i>Lycopersicon esculentum</i> Mill.	6	DS, FR, DR
Tobacco	<i>Nicotiana tabacum</i> L.	2	HR, NC, RNC
Rice	<i>Oryza sativa</i> L.	3	HR
Potato	<i>Solanum tuberosum</i> L.	4	IR
Wheat	<i>Triticum aestivum</i> L.	5	HR
Maize	<i>Zea mays</i> L.	23	HR, IR, MS

MS = male sterility, VR = virus resistance, IR = insect resistance, FR = fungi resistance, HR = herbicide resistance, MFAC = modified fatty acid composition, DR = delayed ripeness, MM = modified metabolism, MC = modified coloration, DS = delayed softening, RNC = reduced nicotine content

Europe include oilseed rape (herbicide resistance, male sterility), sugar beet (herbicide resistance), chicory (male sterility), and potatoes (starch modification). Since introduction of GMPs in the practice of agriculture, the effects on agronomy and the ecological risks were intensively discussed and investigated. The ecological issues of the release and use of GMPs can be described generally as follows:

- probabilities of harm focus on weediness (for instance, the invasion of weeds or the weediness of crops),
- spread of the transgene by vertical gene flow (outcrossing, gene transfer from GMP to another crop, gene transfer from GMP to wild plants),
- spread of the transgene by horizontal gene flow (for instance, from GMPs via pollen to insects or from GMPs to bacteria), and
- potential of any unintended or pleiotropic effects (for instance, changes of fitness of the crops).

From the above-mentioned ecological issues the gene flow was investigated in several experiments under lab as well as under field conditions. It can be stated that gene flow is strongly influenced by the biology of the species and is likely to vary with different breeding systems and modes of pollination. The opportunity for natural hybridization between species in nature depends on many pre- and post-zygotic factors (table 1.13).

The likelihood of pollen-mediated gene flow depends on the presence of sexually compatible relatives that are found as wild plants and arable weeds. For that reason, under European conditions

TABLE 1.13
Factors Determining the Likelihood of Hybrids Between Crop Plants and Related Species Becoming Established in Agricultural or Natural Habitats ^{13,45}

The production of viable hybrid seeds	
1.	Compatibility of the two parental genomes (mitotic and genetic stability)
2.	Ability of the endosperm to support hybrid embryo development
3.	Direction of the cross
4.	Number and viability of hybrid seeds
Establishment of hybrid plants from seeds in soil	
5.	Seed dormancy
6.	Vigor of the hybrid plant
7.	Direction of cross: maternal effects influencing seedling vigor
8.	Nature of habitat: wild, semi-wild, or agricultural
9.	Nature of competition from other plants
10.	Influence of pest, disease, and animal predators
Ability of the hybrid to propagate vegetatively and sexually	
11.	Method of vegetative propagation
12.	Persistence of vegetative propagules in agricultural habitats
13.	Dissimination of vegetative propagules
14.	Invasiveness of vegetative propagules in natural habitats
15.	Sexual breeding system: cross-compatible, self-compatible, ability to cross to either parental species
16.	Male and female fertility: meiotic stability and chromosome pairing
17.	Seed number and viability
18.	Seed dormancy
19.	Nature of habitat: wild, semi-wild, or agricultural
20.	Nature of competition from other plants
21.	Influence of pest, disease, and animal predators

the frequency of gene flow from outcrossing to wild relatives is low in cereals, potatoes, and legumes (table 1.14).

1.2.6 PRECISION FARMING

Precision farming is a site-specific crop production management system that pursues the following aims:

- Improvement of crop yields and quality of harvest products,
- Reduction of costs for crop cultivation,
- Reduction of environmental pollution, and
- Improving the documentation of cultivation techniques.

Precision crop production is based on identifiable management units in the fields taking account the local soil and ecological conditions. Generally three concepts exist to realize precision farming:

1. Mapping approach, also called as GIS-overlay,
2. Real-time sensor approach, and
3. Real-time sensor approach together with map-overlay.

The traditional approach to soil fertility and crop management has been to treat fields as homogeneous areas. Fertilizers, seed amounts for sowing, and pesticide doses are calculated on a

TABLE 1.14
Frequency of Pollen-Mediated Gene Flow in Different Crop Types⁴⁵

Crop	Frequency of gene flow from outcrossing	
	Crop to crop	To wild relatives
Oilseed rape	High	High
Sugar beet	High (seed), low (crop)	High (seed), medium (crop)
Maize	Medium to high	No known wild relatives
Potatoes	Low	Low
Wheat, barley, oats	Low	Medium (oats), low (wheat & barley)
Rye, rice	Medium	Low (rye), high (rice)
Legumes	Low	Low
Grasses	Medium to high	High to medium
Vegetables	High (seed), low (crop)	High (seed), medium (crop)
Fruits (strawberries, apples, grapevines, and plums)	Medium to high	Medium to high
Raspberries, blackberries, black currant	Medium to high	Medium to high

whole-field basis. It is known that fields and crop stands are not homogenous. A high spatial variability of soil properties and crop stands as well as plant diseases and crop yields can be observed across the fields. Since new technologies such as the global positioning system (GPS) were introduced, this spatial variability can be described accurately by yield-based management zones or grid sampling strategies.¹⁸ With the mapping approach the fields will be virtually divided into micro plots by using geographical coordinates. Every micro plot gets an application value. For instance, potassium or phosphorus contents of the soil are taken into account during mineral fertilization. The real-time sensor approach can be defined as an online concept in which specific sensors are used to collect information data about the soil or about the crop plant growth. For this concept geographical coordinates are not necessary. This system will be used to apply nitrogen fertilizer, herbicides, fungicides, or growth regulators. The third concept combines both the real-time sensor approach and the map-overlay. With this system precise and efficient cultivation management can be reached.

An example for applying precision farming in the practice of crop cultivation is focused on nitrogen application, which can be executed by using chlorophyll fluorescence measurements.⁷ It is a noncontacting method to observe the N-status of the plants by using laser-induced chlorophyll fluorescence detection. It is expected that the chlorophyll fluorescence spectra of the plant can be used for fast determination of the leaf chlorophyll content and as an indicator of the relative concentration of N. The main advantage of these laser-based fluorescence sensors compared to passive sensors is the possibility of measurement almost independent of light conditions. This method can be applied in wheat and maize. Field experiments with these crops have shown that the fluorescence ratio F690 nm/F730 nm was inversely correlated with N content, N uptake, and plant biomass.⁷ The results indicate that nitrogen uptake can be reliably detected through chlorophyll fluorescence measurements under field conditions.

Another example for applying precision farming in the practice of crop cultivation is weed control. The common practice is to apply herbicides uniformly over the whole field. But the herbicide application is not necessary in the weed-free areas. A variable rate application of herbicides according to the weed occurrence can contribute to optimize the use of production inputs and to reduce the input of biocides into the environment.¹⁴ Two systems are developed for automatic weed detection. On the one hand, an image analysis system uses CCD cameras and image analysis software to detect the weed species composition and to discriminate weeds from crop plants based

on color, shape, and texture features. On the other hand, optoelectronic sensors measure the reflectance of light in a certain range of wavelengths. A system of site-specific weed control in sugar beet, maize, winter wheat, and winter barley was developed in Germany.^{20,14} This system includes on-line weed detection using digital image analysis, computer-based decision making, and GPS-controlled patch spraying. The economic benefits of this system were high in all crops when type and dosage of herbicides were varied according to weed distribution.

Also at other fields of crop production several specific methods of precision farming are developed. Some of these methods are the use of electromagnetic induction to detect soil properties, the site-specific nitrogen fertilization based on remote sensing and simulation, the use of a mechanical sensor called a pendulum-meter for scanning the crop biomass and models for site-specific seeding rates in cereals.

Generally it can be concluded that the technical developments of the last years led to strong changes in crop management. These developments are dominated by the economic pressure to increase the production efficiency. On the other hand, precision farming systems can also contribute to reduce the environmental impacts of agricultural land use and to increase the transparency in the agricultural production process.

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2 Agrodiversity: Genetic Diversity in Crops and Cropping Systems

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2.1 DIVERSITY AND PLANT BREEDING

For thousands of years mankind has altered the genomes of crop plants to improve their yields and qualities, first by domestication of suitable plants and selection of landraces, followed by classical plant breeding strategies during the last 150 years, later complemented by cell- and tissue-culture techniques, and most recently by molecular breeding. For many of today's crop plants domestication started 9,000 to 13,000 years ago by selection of wild plants with certain traits meeting the needs of humans.¹ The repeated cultivation and maintenance of these selected plants under site-specific conditions led to landraces highly adapted to particular growing conditions and production methods. In some areas with extremely harsh conditions these landraces are still of certain relevance. Starting in Europe in the first half of the 19th century, landraces were in many parts of the world gradually replaced by higher yielding cultivars generated by classic breeding strategies.² These modern plant breeding strategies have led to a multiplication of yield and considerable quality improvements in many important crop plants. At the same time the continuous selection for specific traits and higher yields has caused a loss in diversity in modern breeding material compared to landraces or the wild ancestors of cultivated plants. The domestication and breeding process usually represents a kind of genetic bottleneck. On the other hand, diversity is a precondition for breeding. Without a broad base of heterogeneous plant material it is impossible for plant breeders to produce cultivars that meet the changing needs regarding adaptation to growing conditions, resistance to biotic and abiotic stresses, or quality requirements. Therefore, the most efficient way to further improve the performance of crop varieties is still to have access to a large and diverse pool of genetic variation.

2.2 MEASURING MOLECULAR DIVERSITY

For the determination of diversity on a molecular level several different marker systems have been established during recent decades. The very first commonly used molecular markers were isozymes

TABLE 2.1
Overview of the Most Commonly Utilized Molecular Markers for the Assessment of Diversity in Crop Plants

	Isozymes	RFLPs	RAPDs	AFLPs	SSRs
Codominance	yes	yes	no	no	yes
Number of loci	low	high	high	high	high
Level of polymorphisms	low	medium	high	high	high
Reproducibility	high	high	low	high	high
Technical demands	low	medium	low	medium	medium

followed by various DNA-based markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms), and SSRs (simple sequence repeats; microsatellites) (table 2.1).

2.2.1 ISOZYMES

Isozyme (or isoenzyme) markers are variants of enzymes with identical functions in a specific species that can be separated and visualized by electrophoresis.³ The isozyme markers encompass enzyme variants that are the product of different loci (isoenzymes) as well as enzymes that are the product of different alleles of the same gene (alloenzymes). For the identification of polymorphisms in isozymes crude protein extract is separated by electrophoresis and visualized by subsequent enzymatic staining. The assay is relatively quick and easy to carry out. On the other hand, the number of markers as well as the level of polymorphisms is relatively low. In the beginning of diversity analysis on a molecular level, isozyme markers were frequently used in a large number of crop species (e.g., barley, rice, maize, and common bean).^{4–8}

2.2.2 DNA MARKERS

Because of their larger number of loci and a higher level of polymorphisms, DNA markers soon became more important than isozyme markers. While RFLPs are based on the Southern hybridization technique, RAPDs, AFLPs, and SSRs are PCR-based marker systems.

RFLP analysis is an application of the southern blotting procedure and was first described by Botstein et al. in 1980.⁹ Genomic DNA is digested with appropriate restriction endonucleases and the generated fragments are electrophoretically separated, transferred to a membrane and hybridized to a specific cloned DNA sequence either derived from genomic DNA or cDNA. The obtained length polymorphisms of the DNA fragments are either due to deletions and insertions or to point mutations in the recognition sites of the restriction enzymes. The codominantly inherited RFLP markers have successfully been used for the assessment of genetic diversity in various plant species (e.g., maize, oilseed rape, *Brassica campestris*, tomato, and cucumber).^{10–15}

RAPDs are DNA fragments amplified by PCR using short random primers.¹⁶ RAPDs are very easy and quick to assay, but show relatively low reproducibility between different laboratories and are of dominant inheritance. RAPD markers have been successfully used to determine genetic diversity, for example, in wheat, barley, and the common or Lima bean.^{17–21}

The AFLP technique is a combination of restriction digestion and PCR amplification.²² After digestion with restriction enzymes, adaptors are ligated to the ends of the restriction fragments. In a subsequent PCR reaction with primers complementary to the adaptors but with a 3' overhang, a subset of these restriction fragments is amplified and visualized after separation on a sequencing gel. AFLPs show a high genomic abundance and generate a large number of informative bands per

reaction, but are technically demanding and expensive. AFLPs have been utilized to access diversity in a wide range of crop species (e.g., wheat, soybean, and sunflower).^{23–25}

SSRs or microsatellites are tandem repeats of a very short nucleotide motif (one to five basepairs) that can be amplified with PCR primers specific to the flanking regions of these repeats. SSRs are easy and fast to assay and are robust and highly reproducible. SSRs have become an important tool in crop germplasm management and have been used, for example, to evaluate cultivar variation in rice, soybean, and barley.^{26–28}

2.3 GENETIC DIVERSITY IN MODERN BREEDING MATERIAL

In general the level of genetic diversity within a population of a certain plant species depends on the size of the population, potential selection pressures being imposed on it, the number of reproductive cycles it has gone through, and whether the species is an out-breeder (cross-fertilization) or an in-breeder (self-fertilization). In the following two chapters the genetic diversity within two crop species that differ (e.g., regarding their history as a crop plant and their outcrossing rate) are described. Rapeseed is a partially out-crossing species, where pollination is accomplished by insects, whereas barley is predominantly a self-pollinating plant. While rapeseed has a very recent history as a crop plant with early records originating from the middle ages, barley is known as one of the world's oldest crops.

2.3.1 BARLEY (*HORDEUM VULGARE*)

Barley (*Hordeum vulgare* ssp. *vulgare*) is one of the oldest and most important crop plants. Due to its adaptability to a broad range of growing conditions, barley is one of the world's most widespread cereals. In 2004 up to 57.0 million hectares were planted, with barley gaining an average yield of about 2.72 tons per hectare.²⁹ In a few regions like Ethiopia, Peru, and Tibet, barley is still mainly used for food. However, by far the greatest share of the world's barley production is used for animal feed (about 85%), followed by malting barley for beer and whisky production.

Wild barley (*Hordeum vulgare* spp. *spontaneum*) is the progenitor of today's cultivated barley. The domestication process, like for many other of today's crop plants, started between 10000 and 8000 BC in the region of the Fertile Crescent.^{30–31} This region in the Middle East was first suggested to be the center of origin by Vavilov and later by Harlan because of the broad diversity of wild as well as cultivated barleys and archeological remains of barley grains found in this region.^{32,30} This idea was lately supported by a survey by Badr et al.,³³ who studied 400 AFLP polymorphic loci in 317 wild and 57 cultivated barley lines and found that the wild populations from Israel–Jordan are more molecularly similar than are any others to the cultivated gene pool.

During prehistoric time the cultivation of barley expanded. Starting from the Middle East, the neighboring regions Anatolia and Iraq were reached first. There is archaeological evidence that barley was cultivated during the six millennium BC in Greece and one millennium later in Spain and the Lower Rhine region. During the same period of time barley cultivation expanded to the Mediterranean and North African coastal regions and Ethiopia. In prehistoric Egypt barley was the most important cereal plant.²

The first domesticated barleys distributed during this process were two-rowed and covered forms. Naked as well as six-rowed barleys first appeared by 6500 BC in the regions of today's Israel and Iraq, respectively.³⁴ One of the most important traits for cultivation was the nonbrittleness of rachis to ensure a efficient harvest of grains. In addition to high productivity of seed material, different quality characteristics and tolerance to various types of biotic and abiotic stresses might have been important for selection during the migration process and changed various plant characteristics irreversibly as barley became a cultivated crop.³⁵

Barley was first brought to America by Columbus during his second voyage in 1493, but remained of minor importance in the New World for a long time. Nowadays the U.S. and Canada contribute to the world's acreages with about 5.7 million hectares, whereas about 0.9 million hectares of barley production in South America is from a global point of view of minor importance.²⁹

An intensive phase of barley breeding during the first half of the 19th century led to the gradual replacement of the established landraces. In the United Kingdom, France, Germany, and the Danube monarchy the performance of the breeding material was considerably improved by means of single plant and mass selection from landraces. Further improvements in quality and yield were made by the utilization of recombination through cross-breeding. In the agriculturally active regions in central and northwestern Europe landraces disappeared during the early decades of the 20th century and were replaced by high-yielding cultivars.³⁶

The magnitude of the genetic gain achieved by these intensive breeding activities was determined in several studies and varies on average between $16 \text{ kg ha}^{-1} \text{ y}^{-1}$ for the period between 1920 and 1984 in the United States and $74 \text{ kg ha}^{-1} \text{ y}^{-1}$ between 1960 and 1980 in Italy.^{37–43} First results of field tests carried out on 64 six-rowed and 49 two-rowed winter barley cultivars that were registered in Germany during the last 40 years estimate the genetic gain in yield at $54.6 \text{ kg ha}^{-1} \text{ y}^{-1}$ ($r^2 = 0.567$) for the six-rowed cultivars and at $37.5 \text{ kg ha}^{-1} \text{ y}^{-1}$ ($r^2 = 0.621$) for the two-rowed cultivars.⁴⁴

The influence of the domestication process, followed by the distribution of barley from its center of origin to all parts of the world and the recent intensive breeding process on the diversity of barley, can be accessed by means of molecular markers. A number of studies have been carried out to estimate the diversity within and between wild barley populations, to compare the genetic diversity in wild and cultivated barley and assess the diversity within today's modern, high-yielding barley cultivars.

Several studies found a high genetic diversity in today's wild barley populations originating from the Fertile Crescent and its neighboring regions by means of isozymes and different DNA markers. The level of genetic diversity was higher within populations than between regions or between populations within a region, and in many cases genetic diversity was associated with ecogeographical and climatic conditions in such a way that wild barley populations were more diverse in regions with climatically more stressful conditions.^{45–48}

In most of the studies comparing the genetic diversity of cultivated barley with the currently found genetic diversity in its wild progenitor, the diversity within the gene pool of cultivated barley was lower. Analyzing SSR polymorphisms in 207 accessions of wild and cultivated barley, Saghai-Maroo et al.⁴⁹ found significantly higher diversity in wild barley than in cultivated barley at three of the four loci under investigation. An analysis with 16 RFLP probes covering nearly all barley chromosomes showed a higher number of polymorphisms in the wild barleys than in the cultivated barleys. These analyses supported earlier results obtained by a study of ribosomal DNA spacer-length polymorphisms at two loci.⁵⁰ For 1,496 cultivated and 32 wild barleys collected in Tibet the investigation of four esterase loci for isozyme polymorphisms showed a higher degree of genetic diversity in the wild than in the cultivated material at all four loci.⁵¹

To estimate the genetic diversity present in the gene pool of today's modern high-yielding barley cultivars several studies have been carried out on cultivars originating mainly from North America and Europe. Results of an analysis on 104 accessions of cultivated barley from all major barley growing areas of the world tested with four SSR primer combinations showed an average diversity index of 0.56 with a range between 0.11 and 0.94 for the single loci.⁴⁹ In 28 North American spring barley cultivars that were analyzed by means of 100 genomic as well as cDNA-derived RFLP probes, 57% of the cloned sequences detected polymorphisms. The average DI based on the probes that detected polymorphisms was assessed as 0.419 for the genomic probes and 0.762 for the cDNA-derived clones.⁵² In an analysis of 25 European spring barley cultivars with 681 AFLP markers derived from eight primer combinations, 62.1% of the markers were polymorphic. The genetic similarity among all genotypes was extremely high compared to other studies and ranged between

TABLE 2.2
Genetic Diversity (DI⁵⁶) of Six- and Two-Rowed German Winter Barley Cultivars in Relation to the Year of Release

	1959–2003	< 1985	1985–1995	> 1995
Six-rowed cultivars	0.434	0.432	0.419	0.392
Two-rowed cultivars	0.418	0.320	0.407	0.422

0.901 and 0.978, with an average of 0.932.⁵³ In contrast, Casas et al.⁵⁴ investigated 37 European spring and winter cultivars with 32 RFLP probes combined with three restriction enzymes for polymorphisms and found an average genetic similarity of 0.70 between the cultivars with a range between 0.533 and 0.957. In a set of 48 spring and winter cultivars that were registered between 1925 and 1988 in Germany, an analysis with 23 RFLP probes in combination with three restriction enzymes found 43% of polymorphisms in the whole set and 34% and 30% in subsets of spring and winter barleys, respectively.⁵⁵

To get an even more detailed insight into the influences modern plant breeding had on the genetic diversity during the last four decades, 64 six-rowed and 49 two-rowed cultivars that were registered during the last 40 years in Germany and gained certain importance during this period of time were analyzed using a set of 30 SSR markers distributed evenly over the whole genome.⁴⁴ The 30 SSRs corresponded to 169 different alleles and the genetic similarity was estimated on average at 0.51 on the whole set and at 0.56 and 0.58 for the six-rowed and two-rowed cultivars, respectively. In accordance to these results both subsets of cultivars showed on average a similar level of genetic diversity with a DI of 0.434 for the six-rowed and 0.418 for the two-rowed cultivars (table 2.2). By grouping the cultivars according to their year of release, differences in the changes of genetic diversity between the two subsets were found. While the genetic diversity stayed constant over time in the six-rowed material, the diversity of the two-rowed cultivars was increased over the last decades. These results show, that modern plant breeding not only caused a considerable gain in yield in German two-rowed winter barley cultivars, but at the same time increased the genetic diversity found in this crop.

2.3.2 RAPESEED (*BRASSICA NAPUS*)

The rapeseed plant (*Brassica napus* L., genome AACC, 2n = 38) originated through spontaneous interspecific hybridization between turnip rape (*B. rapa* L., syn. *campestris*; genome AA, 2n = 20) and cabbage (*B. oleracea* L.; genome CC, 2n = 18), resulting in an amphidiploid species comprising the full chromosome complements of its two progenitors (cf. fig. 2.1). Because no wild *B. napus* forms are known, it is assumed that the species arose relatively recently, in the Mediterranean region where both of its two parental species concurred. The occurrence of spontaneous chromosome doubling in crosses among closely related *Brassica* diploid species is well documented; the related amphidiploids Indian or brown mustard (*Brassica juncea*; genome AABB, 2n = 36) and Abyssinian or Ethiopian mustard (*Brassica carinata*; genome BBCC, 2n = 34) arose in the same manner after crosses of black mustard (*Brassica nigra*, genome BB, 2n = 16) with *B. rapa* and *B. oleracea*, respectively. Rapeseed (*B. napus*) is today the most widely cultivated crop species in the crucifer family (*Brassicaceae*). The species is divided into two subspecies, comprising on the one hand the swedes (*B. napus* ssp. *napobrassica*) and on the other hand *B. napus* ssp. *napus*, which includes winter and spring oilseed, fodder, and vegetable rape forms. The latter include the distinct leaf rape forms (*B. napus* ssp. *napus* var. *pubularia*), which used to be common as a winter-annual vegetable in many parts of the world.⁵⁷

Brassica vegetables and oilseeds were among the earliest plants to be systematically cropped by mankind. There are indications that a vegetable crucifer was widely cultivated as early as 10,000

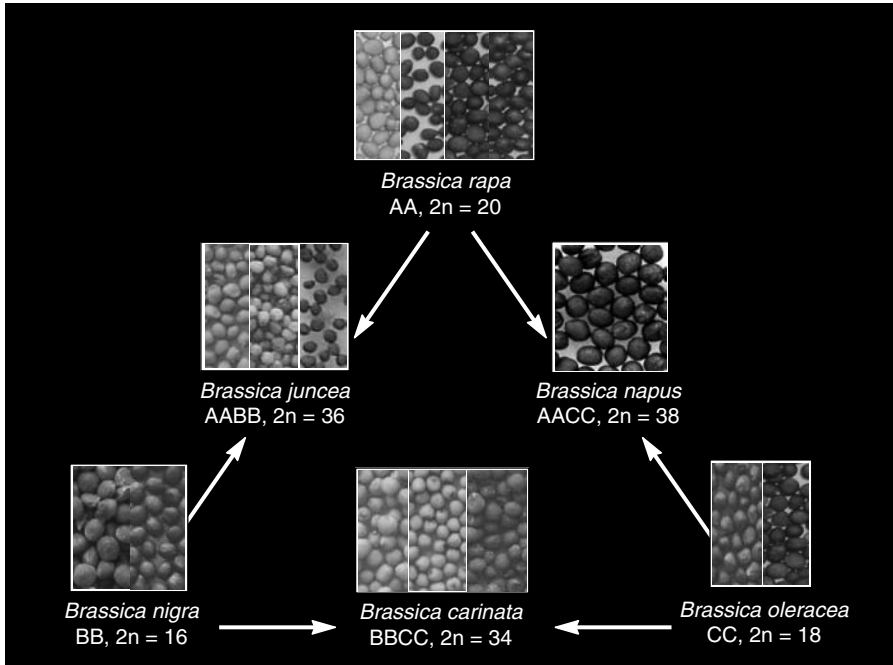


FIGURE 2.1 (See color insert following page 170) Scheme showing the origin of the amphidiploid species rapeseed (*B. napus* L.), *B. juncea* and *B. carinata* via natural interspecific hybridization between progenitor *Brassica* species *B. nigra*, *B. rapa*, and *B. oleracea*, respectively. Genetic variation within species is exemplified by different seed colors associated with variable conformation of the seed coat (e.g., fiber content and structure).

years ago. In India records have been identified that suggest that oilseed brassicas (probably *B. rapa*) were being used as early as 4000 BC, and 2,000 years ago their use had spread into China and Japan. Swedes (*B. napus* ssp. *napobrassica*) were known in Europe at the time of the Romans, and utilization (probably of *B. rapa*) for oil purposes in northern Europe is thought to have begun around the 13th century. By the 16th century, rapeseed was the major source of lamp oil in Europe, although it was not until the 18th century that significant cultivation of the crop was recorded.⁵⁷ Today, oilseed rape is the most important oilseed crop in Europe and only soybean has greater importance worldwide. Oilseed rape production is dominated by China (7.3 million hectare in 2004), Canada (4.9 million hectare), and Western Europe (Germany: 1.3 million hectare), but the crop is also significant in Australia (1.1 million ha), the Indian subcontinent, and Eastern Europe.²⁹ However, oilseed rape has become a major international crop only over the course of the past three decades.⁵⁸ The use of rapeseed oil for lamp fuel was largely superseded by petroleum from the end of the 19th century onward, and only the high quality of rapeseed fats as lubricants for industrial machinery guaranteed continued production of the crop throughout the 20th century. Oil from early rapeseed varieties contained a high quantity of erucic acid (*cis* 13-docosenoic acid, 22:1n-9), which in high doses can lead to cardiac damage and related health problems. Erucic acid also has a bitter taste, meaning that the oil was used only by the poor as a food oil. In times of poverty and crisis, of course, such negative aspects tended to be outweighed by necessity; hence rapeseed production peaked significantly during the wars in Europe in the 20th century, particularly in World War II when rapeseed oil was used, especially for the production of margarine. The poor reputation of rapeseed oil as a foodstuff was overcome only by the development of “0” and “00” rapeseed varieties in the 1970s. The first major breakthrough came with the initial “0-quality” cultivars with erucic acid levels of less than 2%.⁵⁷ Earlier rapeseed cultivars contained up to 50% erucic acid in the seed

oil. The identification of the fatty acid mutants from which the first 0-rapeseed originated was made possible by major improvements in seed analysis techniques. The first erucic acid-free variety, derived from a spontaneous mutant of the German spring rapeseed cv. "Liho," was released in Canada in the early 1970s. The value of the crop was still suppressed by the presence of high quantities of glucosinolates in the seed, however, which made rapeseed cake or meal, the residue of oil extraction, unsuitable as a feed for monogastric animals since the digestion of glucosinolates results in the release of toxic by-products. In 1969 the Polish spring rape variety "Bronowski" was identified as a low-glucosinolate form, and this cultivar provided the basis for an international backcrossing program to introduce this polygenic trait (at least three recessive genes for low glucosinolate content) into high-yielding erucic acid-free material. The result was the release of the first 00-quality spring rapeseed variety, "Tower," in 1974 with very low erucic acid and low glucosinolate content, and thus began the advance of oilseed rape (canola) in the following decades to one of the most important oil crops in temperate regions. The particular value of rapeseed oil lies in its diverse range of uses. Besides its use as a highly nutritional food oil, rapeseed oil also provides a raw material for an astounding array of products ranging from rapeseed methyl ester (biodiesel) to industrial lubricants and hydraulic oils, tensides for detergent and soap production, and biodegradable plastics. Modern rapeseed oil, with its valuable fatty acid profile obtained from 00 or canola cultivars, is used as a basis of margarine or salad oil for human consumption. Both high content of monounsaturated oleic acid (C18:1) and polyunsaturated alpha-linolenic acid (C18:3) determine the high nutritional value of modern rapeseed oil. Nevertheless, this oil is also used for processing hydraulic or lubrication oil and for biodiesel (methylesters of rapeseed oil) production. Still, the rapeseed cake or meal is mainly used for feeding ruminants since its glucosinolate and crude fiber contents limit the use for feeding monogastric animals like chickens or pigs.

Oilseed rape is cultivated in Europe and Asia predominantly as a winter form, whereby in Canada, northern Europe, and Australia only spring forms are suitable. The differentiation into winter and spring forms is governed by a genetic mechanism controlling the requirement for vernalization to promote the onset of flowering. Spring oilseed rape does not require vernalization and is not winter-hardy, hence the crop is sown in spring and stem development begins immediately after germination. Winter oilseed rape, on the other hand, is sown in autumn and survives the winter in a leaf rosette form on the soil surface. Shooting and flowering generally occurs in late spring, with pod development and ripening taking place over a period of around six to eight weeks until mid-summer. As a member of the *Brassicaceae* family (*Cruciferae*), *B. napus* possesses a typical radial flower comprised of four petals. The inflorescence is racemose, with indeterminate flowering beginning at the lowest bud on the main raceme and continuing upward during the following days. The stigma is receptive from about three days prior until three days after the opening of the flower. The normally yellow flowers have one pair of lateral stamens with short filaments and four median stamens with longer filaments. Oilseed rape anthers of the four long stamens become extruded after the flower buds open. In contrast to the majority of *B. rapa* and *B. oleracea*, its diploid progenitors, *B. napus* is a facultative outcrossing species with a high degree of self-pollination. When insect pollinators (bees, bumble bees, a.o.) are abundant a greater proportion of cross-pollination can occur, although through targeted fertilization-direction it is possible to obtain up to 100% outcrossing (e.g., using male-sterility systems for hybrid varieties). The world average rapeseed yield of about 1.58 metric tons per hectare covers a wide range, from 2.93 t/ha in Western Europe, 1.61 in Australia, 1.58 in China, and 1.42 in Canada to 0.87 t/ha in India.²⁹ These differences are due to variety (e.g., winter vs. spring type), climate, and soil as well as agricultural inputs (seed quality, fertilizers, and agrochemicals) and different agronomic techniques.

After Morinaga⁵⁹ and U⁶⁰ discovered through cytogenetic studies in the early 1930s that amphidiploid *Brassica* species originate from diploid progenitors and contain the complete chromosome sets of their parental species, chromosome studies came to play a leading role in genome analysis among the *Brassicaceae*. The age of classical cytogenetics has, however, been largely superseded by the implementation of DNA techniques during the past few decades, and the

difficulties associated with *Brassica* chromosomes as a cytological object—in particular their small size and lack of distinctive cytological landmarks—have made *Brassica* cytogenetics a rare art among the proliferating molecular marker technologies. For many years little more could be achieved than simple chromosome counts or meiotic studies of the offspring from interspecific or intergeneric crosses, giving insight into genome homologies among the various *Brassica* relatives. In recent years, however, advances in the molecular cytogenetic technique of fluorescence *in situ* hybridization (FISH), which enables the direct chromosomal localization of labelled DNA probes, have enabled a resurgence of cytogenetic analyses in plant genome research and molecular breeding. *Brassica* cytogenetics dates back to the early decades of the 20th century, when a number of predominantly Asian scientists began with detailed investigations of chromosome numbers and chromosome pairing in some of the important crucifer species. The first major achievement was the publication of the chromosome number for *B. rapa* by Takamine in 1916, followed by the synthesis and analysis of *Raphanobrassica* by Karpechenko in 1927.^{61–62} However, it was the work of Morinaga and U that gave rise to another generation of researchers who began to look more deeply into genome homology in the *Brassicaceae*.^{59–60} The development of ovary culture and embryo rescue techniques in the 1950s enabled enormous progress in the study of genome homologies based on chromosome pairing analyses. Additionally, technological advances in optical equipment and microscopy brought a great improvement in cytological techniques in general. Based on these techniques Röbbelen was the first to publish detailed cytological descriptions of *Brassica* somatic chromosome structure in 1960.⁶³ From a cytogenetics perspective the period between the 1960s and the end of the 1980s was dominated by an intensive effort to collect and classify botanical representatives of the crucifer tribe and to study the evolutionary and genomic relationships among this array of species. One of the major personalities in this movement was Harberd, whose study of chromosome pairing among a huge number of species eventually led to the classification of cytodelmes describing homologous genomes.⁶⁴ Based largely on this work, we know now that there is extensive genome homology or homoeology throughout the entire *Brassica* coenospecies, and from a plant breeding perspective in particular it has become well known that we consequently have the possibility to broaden gene pools for the introgression of novel genes or alleles, well beyond the species boundary. Related *Brassica* species and their relatives among the *Brassicaceae* represent a huge pool of potential gene donors for agronomically relevant traits in oilseed rape.

Fluorescence *in situ* hybridization (FISH) techniques offer the potential not only for more reliable chromosome identification in *Brassica*, but also in terms of the information they might be able to offer regarding the integration of genetic and physical maps, for ordering molecular markers and measuring physical genome distances, and for structural and functional chromosome analysis. FISH methods for the accurate localization of repetitive DNA sequences at chromosomal subarm level, particularly ribosomal DNA sequences, have enabled the elucidation of karyotypes for *B. napus* and its progenitor species and the identification of A and C genome chromosomes in the amphidiploid species (fig. 2.2).^{65–67} FISH hybridization of BAC clones to *B. oleracea* and *B. rapa* chromosomes represents a first step toward integration of physical and genetic maps with the karyograms of the diploid species and their amphidiploid hybrid *B. napus*.^{68–69} Such comparative fiber-FISH mapping results support evidence that chromosomal duplications, rather than regional expansion due to accumulation of repetitive sequences in the intergenic regions, played a major role in the evolution of the diploid *Brassica* genomes. The use of total genomic DNA as a FISH probe (genomic *in situ* hybridization, or GISH) is especially useful for diagnostic studies of the amount and integration of foreign chromatin in interspecific and intergeneric plant hybrids.⁷⁰ Hybrids between high-yielding rapeseed cultivars and related species are relatively easily produced and have often been used to develop new lines containing introgressed traits like novel pest or disease resistances. Great advances in interspecific hybridization have resulted from the application of *in vitro* techniques for the generation of viable offspring from interspecific and intergeneric hybrids.⁷¹ Identification of alien DNA in wide crosses has been achieved by quantification of chromosome content by flow cytometry and by tracing chromosome and DNA transfer using

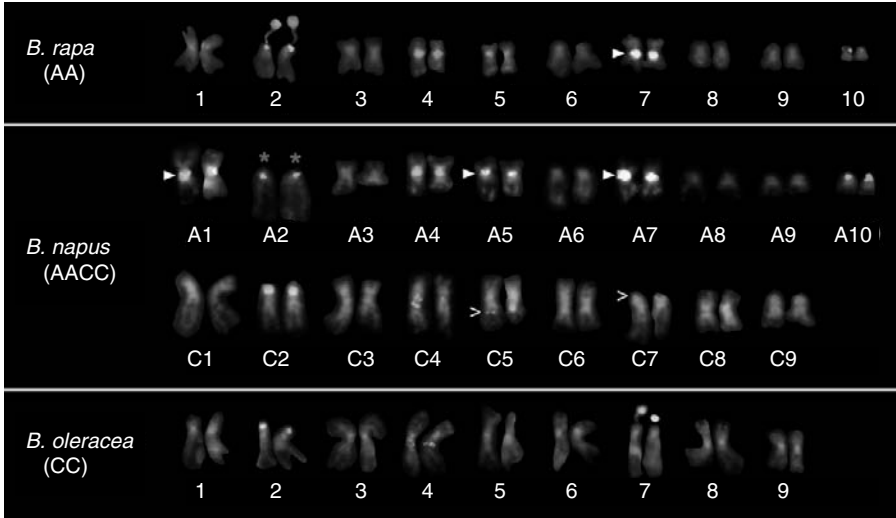


FIGURE 2.2 (See color insert) Karyotypes of rapeseed and its parental species based on fluorescence *in situ* hybridization patterns with 5S (green) and 25S (red) rDNA probes and DAPI staining (blue), for *B. rapa* L., *B. oleracea* L. and their amphidiploid *B. napus* L.⁶⁷

molecular markers.⁷² Visualization of alien chromatin in interspecific hybrids using *in situ* hybridization techniques, on the other hand, potentially enables pinpointing of introgressions to specific chromosomes.^{70,73}

Intergeneric sexual hybrids between *B. napus* and *Sinapis arvensis* containing novel genes for resistance against blackleg disease on chromosome additions and introgressions were analyzed via GISH by Snowdon et al.⁷⁴ Selfed BC₃ progenies included fertile plants exhibiting high seedling and adult plant resistance associated with the presence of an acrocentric addition chromosome from *S. arvensis*. Furthermore, some individuals with adult plant resistance but cotyledon susceptibility were observed to have a normal *B. napus* karyotype with no visible GISH signals, indicating introgression lines carrying at least a subset of the *S. arvensis* resistance genes. Schelfhout et al. used a B-genome-specific centromeric repeat sequence as a PCR and FISH marker to characterize B-genome introgressions in sexual progeny from *B. napus* × *B. juncea* crosses exhibiting various traits of agronomic interest, including resistance against blackleg disease and pod shattering.^{75–76} Genotypes with normal *B. napus* karyotype were identified in which the minisatellite sequence could be detected by PCR, although no FISH signals were observed, indicating small chromosomal introgressions that carried the gene of interest. Voss et al.⁷⁷ generated intergeneric crosses between spring oilseed rape and nematode-resistant oil radish (*R. sativus*) genotypes, using embryo rescue to overcome incompatibility barriers. In three backcross (BC) generations, highly resistant progeny with a minimal number of *R. sativus* chromosomes were selected by resistance testing accompanied by GISH analysis. This strategy led to the identification of a resistant BC₃ plant with a monosomic, acrocentric addition chromosome. This individual was backcrossed once again to produce a stable disomic addition line, however, efforts to introgress the resistance on a stable introgression failed. Similarly, Peterka et al.⁷⁸ (2004) also generated oilseed rape interspecific hybrid lines containing nematode resistance on a monosomic *R. sativus* addition chromosome. Here, *R. sativus* chromatin was identified by PCR and FISH with a *Raphanus*-specific centromeric repeat sequence. In this case also, however, no intergenomic transfer of the resistance was reported. Fahleson et al.⁷⁹ analyzed somatic hybrids between *Eruca sativa* and *B. napus* using *in situ* hybridization with two *E. sativa*-specific repetitive DNA sequences accompanied by GISH. One of the repetitive sequences showed 100% similarity with a part of the *E. sativa* rDNA intergenic spacer, and localized to the

three pairs of *E. sativa* rDNA loci, whereas the other clone was a tandemly repeated element located close to the telomeres on at least 10 *E. sativa* chromosomes. Analysis of progenies derived from the somatic hybrids revealed the presence of *E. sativa* DNA, however, no intergenomic translocations could be detected by GISH, although the somatic hybrid progeny contained one or two complete *E. sativa* chromosomes. Together these results emphasize the fact that chromosome translocations among nonhomologous genomes are more likely in the presence of homoeologous chromosome pairing allowing intergenomic recombination. Genome homoeology at the chromosomal level is expected to be more extensive between oilseed rape and its closer relatives, and this is confirmed by the relative ease with which agronomic traits have been transferred to *B. napus* from *B. nigra*, *B. juncea*, *B. carinata*, and *Sinapis* species in comparison with the difficulties observed in more distant crosses. On the other hand, successful transfer of genes of interest in intertribal asymmetric hybrids has also been demonstrated on a number of occasions and may indicate unknown or partial genome homologies. Interesting results in this respect were obtained by Wang et al.,⁸⁰ who produced sexual progenies of asymmetric somatic hybrids between *B. napus* and *Crambe abyssinica* in an effort to improve the fatty-acid composition of oilseed rape seed. Through meiotic GISH these authors were able to identify intergenomic chromatin bridges and detect asynchrony between the *B. napus* and *C. abyssinica* meiotic cycles. Lagging, bridging, and late disjunction of univalents derived from *C. abyssinica* were observed, whereas analysis of cleaved amplified polymorphic sequence (CAPS) markers derived from the *FAE1* gene showed novel patterns different from the *B. napus* recipient in some hybrid offspring. This indicated the existence of novel allelic variation in the interspecific hybrids that presumably arose from introgression from crambe chromatin to one or more *B. napus* chromosomes. Some of the recombinant offspring contained significantly greater amounts of seed erucic acid than the *B. napus* parent, demonstrating that it is possible to introgress agronomic traits from distantly related crucifers into elite oilseed rape material. In another example, Winter et al.⁸¹ used mitotic GISH to characterize recombination lines containing genes for blackleg resistance from *Moricandia arvensis*. Resistant lines were identified that exhibited a normal *B. napus* karyotype but carried the *Moricandia* resistance genes on putative chromosome introgressions. Although such crosses can exhibit significant linkage drag and hence must be viewed as extremely basic material from a breeding perspective, such prebreeding is of enormous interest in terms of broadening the genetic variability for particular traits where little variation is available within *B. napus* itself.

Due to its partial allogamy, oilseed rape can be treated as a self-pollinating or outcrossing species. Backcrossing has been successfully used to transfer simply inherited traits such as low erucic acid and glucosinolate content into adapted breeding material. *Brassica napus* is also one of the most amenable crop species to improvement through biotechnology. For instance, it is possible to reproducibly obtain haploid and subsequently doubled-haploid (DH) plants through anther and/or microspore culture (e.g., Weber et al.⁸²). The principle advantage of the haploidy technique is the rapid fixation of segregating genotypes, occurring in lower frequency, in which recessive genes coding for specific traits are combined in the homozygous condition. Thus, utilization of microspore culture can allow a substantial acceleration of the breeding cycle. Due to the generally high response of *B. napus* genotypes, the use of DH production has become common practice in commercial breeding programs and has already resulted in numerous licensed cultivars. Besides haploid techniques, wide hybridizations using embryo rescue techniques or protoplast fusion can also be used to create novel genetic variation. However, once a useful property has been identified in a basic breeding stock (e.g., a mutant line or germplasm from a wild relative), it may take many years to accomplish the development of cultivars possessing this novel desirable trait. Marker-assisted selection has shown a significant impact on the efficiency of plant breeding routines such as backcrossing programs. In cases where conventional approaches have not been sufficient, further improvements can be achieved by genetic engineering.

As crop plant gene pools narrow through continued selection for yield and other agronomic traits, it becomes more and more important for breeders to have suitable and efficient tools for an

effective discrimination of breeding lines. Over the last decades an array of genetic marker systems based on DNA polymorphisms have been developed that potentially improve the efficiency of selection in rapeseed breeding programs by enabling a more accurate definition and exploitation of genetic variation. Many studies have also demonstrated the use of molecular marker techniques for the analysis of genetic variation in crop plant species. In oilseed rape, Becker et al.⁸³ (1995) compared cultivars and resynthesized lines by isozyme and RFLP markers and concluded that RS forms are a suitable resource for broadening the genetic base of the species. Song et al.⁸⁴ described the rapid genome changes in synthetic *Brassica* polyploids and discussed the evolutionary implications arising from the ability of polyploid species to generate extensive genetic diversity in a short period of time. Thormann et al.⁸⁵ used RFLP and RAPD markers to determine genetic distances in and between cruciferous species. Halldén et al.⁸⁶ characterized *B. napus* breeding lines by RFLP and RAPD techniques, while Diers and Osborn compared RFLP patterns in 61 winter and spring rapeseed genotypes and concluded that the two forms constitute two genetically distinct groups.¹² The relationship between genetic distance and heterosis in oilseed rape was investigated by Diers et al.⁸⁷ using RFLP markers and by Riaz et al.⁸⁸ with sequence-related amplified polymorphisms (SRAP). Plieske and Struss were able to clearly differentiate winter and spring rapeseed in a cluster analysis using simple sequence repeat (SSR) markers.⁸⁹ Furthermore, RAPDs were used by Mailer et al.⁹⁰ for discrimination among rapeseed cultivars, and by Demeke et al.⁹¹ for taxonomic analyses in *Brassica* species. Lombard et al.⁹² used AFLPs to genotype winter rapeseed cultivars and to estimate genetic similarities and demonstrated the effectiveness of AFLP markers for genetic distinction among cultivated rapeseed types.

Many cultivars still represent a kind of (pure) lines (also called open pollinated varieties; OP varieties) derived from breeding schemes designed for self-fertilizing crop, (i.e., pedigree selection or modifications thereof). However, an increasing number of cultivars nowadays are single cross-hybrids derived from a male-sterile female and a corresponding fertile male restorer line. For example, the current German list of registered winter rapeseed cultivars comprises 56 varieties, 14 of which are restored hybrids.⁹³ Two leading cultivars represent the line ("Oase") and the hybrid type ("Trabant"), both characterized by a very high oil yield. Both, OP and hybrid cultivars represent an enormous genetic variability, since they can create highly diverse progeny due to genetic segregation, particularly in cases where extremely different parents are used to create novel hybrids. However, breeding for agronomic and economic value tends to bias exploitation of genetic variation. Therefore, it is necessary to continuously integrate diverse plant germplasm into the breeding process to maintain a high level of genetic diversity of the basic rapeseed material. Resynthesis of novel genotypes through artificial crosses between the diploid parents, assisted by embryo rescue techniques, has repeatedly been shown to be a useful strategy for broadening the genetic basis of rapeseed. Both progenitor species exhibit an extremely broad genetic and phenotypic diversity that gives the potential for a huge variety of different RS rapeseed forms.^{94–96} The relatively high extent of intergenomic recombination between A and C genome chromosomes in early generations of RS rape further increases the potential for creation of novel genotypes through resynthesis.^{95,97}

In a detailed study Seyis et al.⁹⁸ have compared RS rapeseed lines originating from crosses between an Indian yellow sarson (YS) and different cauliflower varieties (BK2256, BK3094, and BK3096) using AFLP markers. Genetic diversity was compared to a collection of diverse spring oilseed and fodder rape types from different countries (Australia, Canada, Denmark, France, Germany, Sweden, New Zealand) by principal coordinate (PCO) analysis.

Resynthetic rapeseed lines were generated by embryo rescue using ovule culture from crosses between five varieties of high erucic acid cauliflower (*B. oleracea* L. convar. *botrytis*) and a YS (*B. rapa* ssp. *trilocularis*) cultivar with both high erucic acid and high glucosinolate contents (++) quality). A total of 165 RS lines were tested in field trials together with 40 spring oilseed and fodder rape cultivars from European gene banks. All RS lines are self-fertile, possess traditional ++-seed quality, have moderate or no vernalization requirement, and weak winter hardiness (cf. Seyis et al.⁹⁸).

For AFLP marker analysis a set of three primer combinations was used, scoring a total of 467 polymorphic bands in the complete set of *B. napus* RS lines and spring rapeseed varieties. As expected the RS lines proved to be closely related due to their common *B. rapa* parent YS. The RS combinations BK2256 $\times$ YS, YS $\times$ BK3094, and YS $\times$ BK2256 showed a high genetic similarity, indicating a close relationship between the two cauliflower cultivars BK2256 and BK3094, whereby BK2256 $\times$ YS clustered closer to YS $\times$ BK3094 than to YS $\times$ BK2256. However, the two reciprocal lines are only separated by the third PCO-axis, indicating that their genetic differentiation arose from only a relatively small number of gene loci. Furthermore, the genetic distances separating these three RS families were comparable with the smallest distances between different rapeseed varieties, hence this group can probably be regarded collectively in terms of genetic diversity for breeding purposes. On the other hand, some of the sibling lines from the RS groups YS $\times$ BK3096, as well as YS $\times$ BK2256 and YS $\times$ Venus, were dispersed in the PCO diagram (e.g., RS83, RS158, and RS125), indicating unexpected genetic differences in lines originating from these crosses.

The 40 cultivars studied formed a distinct cluster in the PCO analysis. The old German landrace "Janetzki's" (++) , the Canadian canola-type varieties "Regent," "Tristar," and "excel," and the two German 00-cultivars "Callypso" and "Evita" proved to be most genetically distant from the bulk of the material (fig. 2.3). Also, the fodder type "Moana" from New Zealand (both ++), along with the Australian cultivars "Wesbrook" and "Marnoo" (both 00), were shown to be genetically distinct.

Since only one *B. rapa* parent was used for the development of the RS lines, this novel rapeseed material had been expected to be rather closely related. However, in addition to the molecular data the phenotypical data from the field evaluation of morphologic and agronomic traits including plant height, leaf morphology, days to flowering, flowering period, time of maturity, vegetation period, and seed yield components (pods per plant, seeds per pod, 1,000-seed weight) showed a clear differentiation between the RS families, which in turn could be clearly differentiated from natural spring rapeseed cultivars, fodder types and Canadian oil types. The observation of RS lines in the present study with quite distinct AFLP genotypes to sibling lines from the same cross may be a consequence of intergenomic recombination as reported earlier. In some cases the genetic differences were reflected in a distinct or unusual morphology of the line in question, demonstrating the potential of such genetic variation to manifest itself in novel phenotypic variation.

Altogether, the results of the study by Seyis et al.⁹⁸ and others show that a vast genetic variation exists within spring rapeseed just like in winter rapeseed materials. In addition, both groups represent clearly differentiated genetic pools that form the basis for the identification of superior combinations for the breeding of both line and hybrid varieties. However, the limited geographic range of rapeseed and intensive breeding particularly focusing on canola (00) quality has led to a comparatively narrow genetic basis of current breeding materials. In this context, resynthetic (RS) rapeseed genotypes developed by interspecific hybridization between diverse *B. rapa* and *B. oleracea* accessions have the potential to significantly increase the available gene pool and provide important basic germplasm for further improvements of agronomic traits (e.g., disease and pest resistances, seed yield) and quality features (i.e., oil and meal composition). Furthermore, RS rapeseed is potentially of great value for hybrid breeding, since heterosis effects tend to be higher in crosses of genetically distant versus more related materials. On the other hand, RS genotypes are generally not suitable to be used directly for oilseed rape hybrids, because they usually display inferior seed quality traits such as low seed oil content, high erucic acid (C22:1) in the oil, and high seed glucosinolate contents in the meal (++ quality), as well as other undesirable quality traits derived from one or both of the progenitor parents. The large genetic distance between the RS male lines and existing rapeseed cultivars is of particular interest in terms of increasing potential heterosis through hybrid development. In order to investigate the heterotic potential for yield components, selected RS lines from the material described by Seyis et al.⁹⁸ were used to develop experimental hybrids with male-sterile breeding lines. In field trials at three locations several hybrids based on RS lines gave a higher yield potential compared to check cultivars (fig. 2.3; cf. Seyis et al.,⁹⁹

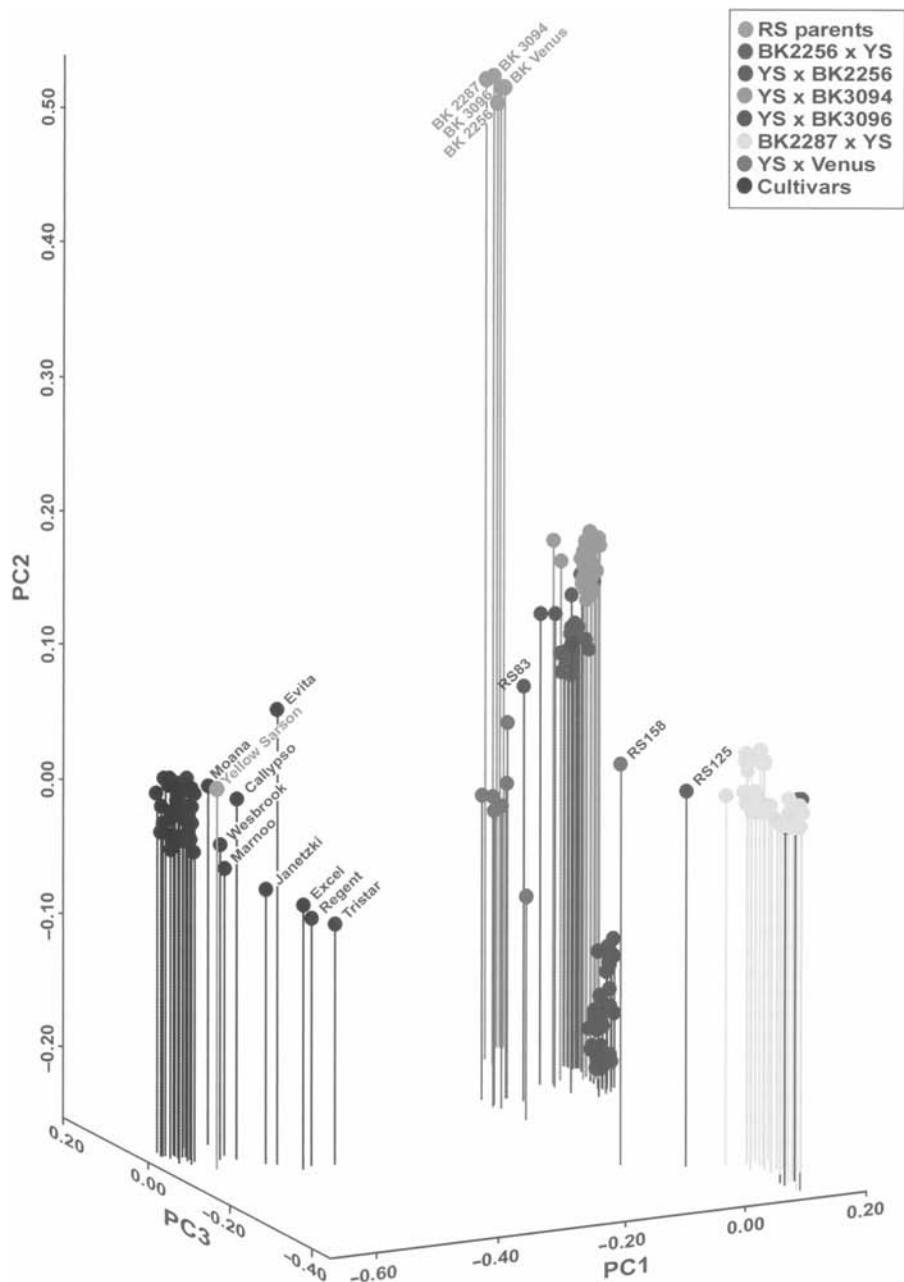


FIGURE 2.3 (See color insert) PCO analysis showing genetic relatedness among a set of spring rapeseed cultivars (black) in comparison with families of resynthesized rapeseed lines (colors).⁹⁸

Spiller et al.¹⁰⁰). No clear differentiation was evident between oilseed and fodder varieties, however, in some cases genetic clusters were observed that corresponded to the original or quality type, especially with more exotic or old material. Based on polymorphisms at RFLP and isozyme loci, Becker et al.⁸³ were able to distinguish rapeseed varieties into summer and winter forms and in terms of their respective geographic origin, particularly in the case of exotic Asian material. Diers and Osborn also distinguished rapeseed genotypes into winter and spring groups using RFLP markers, whereas Plieske and Struss were able to clearly differentiate winter and spring rapeseed

using SSR markers.^{12,89} Of course, many of the polymorphic loci identified in the present study can be attributed to the high genetic distance of the RS lines from the variety material; however, the number of loci polymorphic within the varieties alone was far greater than the number of polymorphic loci in the previous studies. It should be remembered, however, that most fodder rapes originated from the same narrow genetic pool that also gave rise to low glucosinolate (00) oilseed rape forms, and their development and morphology is not necessarily different from that of oilseed forms.

The enormous potential of resynthesis in rapeseed breeding will make efforts dealing with germplasm conservation and well-directed use of the diploid parents more important in the future. On the other hand, the initial yield potential of RS rapeseed (usually spring types) is low. So the use of such forms and the new genetic variability thus created must be directed, particularly with regard to seed quality and yield, to facilitate its integration into high-yielding winter rapeseed breeding material. Specifically, this can be achieved by producing semi-synthetic rapeseed forms via backcrosses to adapted cultivars or by developing high-yielding hybrids. Obviously, the establishment of new gene pools based on novel *B. napus* is limited by its inferior agronomic performance and seed quality (++), hence this approach must be considered under more long-term perspectives. However, the discovery of low-erucic acid mutants among *B. oleracea* accessions and the development of RS rapeseed forms via interspecific crosses with interesting 0- or 00-quality *B. rapa* genotypes will open the possibility to use such new rapeseed material as a genetic resource for quality and yield improvement in oilseed rape.^{96,71,99} As demonstrated, the use of molecular markers such as AFLPs assists in the evaluation of RS lines in terms of describing their genetic distance in relation to existing breeding material, in order to enrich the available gene pool for breeding of this important oil crop species. Based on the broad genetic diversity in the basic *Brassica* materials, including wild species, undomesticated accessions, breeding lines, and released varieties, novel rapeseed cultivars representing a great genetic diversity will continue to be developed in the future.

2.4 CONCLUSION

Genetics-based plant breeding has enabled rapid progress leading to a multiplication of product yield of practically all crop plants. Due to the bottleneck function of domestication and continuous selection for higher yield along with specific resistance and quality traits genetic diversity in modern cultivars has decreased as compared to older varieties, landraces or wild ancestors of the cultivated plants. However, genetic diversity is a precondition for breeding progress regarding major plant characteristics such as adaptation to growing conditions, resistance to biotic and abiotic stresses or quality requirements. Therefore, the creation of novel genetic variation is a major, continuous task of plant breeders to build the basis for further improvements of crop varieties. Plant breeders have many options to widen genetic diversity in respective crop plant species, like barley and rapeseed. In particular novel genetic variation can still be created by sexual hybridisations within and across species, i.e., (a) intraspecific crosses between distinct genotypes within a species i.e., subspecies, landraces, varieties, cultivars or inbred lines, (b) interspecific crosses between related species within a genus leading to fertile hybrids, e.g., *Brassica* sp., *Hordeum* sp., or *Triticum* sp., (c) intergeneric crosses between species belonging to different but related genera, e.g., *Brassica* × *Orychophragmus*, *Brassica* × *Raphanus*, *Festuca* × *Lolium*, *Triticum* × *Secale* (Triticale).

In addition to that, protoplast fusion allows the creation of hybrids which would be extremely difficult to create or not achievable at all by sexual hybridization, e.g., products of intergeneric somatic hybridization between distantly related cereals like rice (*Oryza sativa*) and barley (*Hordeum vulgare*). However, if the distance is too large the fusion product may still be sterile, like in the case of rice–barley or many other hybrids. Again, the results will be more promising in the case of closer related genera or even hybridizations between species belonging to the same genus, like in the case of asymmetric fusions between cells of sunflower (*Helianthus annuus*) and the wild

species *H. maximiliani*, leading to partial hybrids and selected sunflower lines with enhanced resistance against the fungal pathogen *Sclerotinia sclerotiorum*, causal agent of sclerotinia rot.

In addition, genetic engineering allows the creation of new genetic variation since useful genes for plant breeding are already abundantly available. Numerous respective examples include improvements of important agronomic and quality traits. Along with increasing knowledge of plant biochemistry, genetics and physiology the options for broadening genetic variation and plant improvement by breeding are expected to grow substantially.

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3 Soil Space Diversity and Its Dynamics: Qualitative and Quantitative Considerations

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3.1 INTRODUCTION

The heterogeneous structure of nature leads to a huge variety of different habitats for different species and thus, spatial structures are one of the major agents for biodiversity in natural systems. This is true over a wide range of spacial scales, starting from bacteria with a typical size of some 10^{-6} m to populations of elephants with a typical habitat of 10^5 m. The diversity of the related living space is a prerequisite for the coexistence of various species at a given location at the same time.

Besides the local climatic conditions, soil, together with the relief, is one of the key factors that brings spatial structure to natural systems. Jenny [15] postulated that soil is an immediate consequence of some basic soil forming factors, as climate, parent material, relief, and vegetation. Until today, this is a valuable concept and a widely accepted model of soil genesis. This consideration could lead to the conjecture that soil should come out to be rather homogeneous, below the characteristic length scales of the forming factors, which is on the order of at least some 10^1 to 10^3 m. This also corresponds to the typical resolution of soil maps. But if we look closer using a higher spatial resolution, say at the subscale of Jenny's considerations, we become aware that the

structure of soil is tremendously manifold also at the smaller scale. Given a region of homogeneous soil forming factors, we might identify the same soil type everywhere but the variety of features at the subscale is much larger than would be suggested from the corresponding soil map. This is a common experience for those who have ever tried to show students the *typical* soil.

Thermodynamics suggests that structure should vanish with time due to the inherent entropy. However, we are dealing with an open system where the external input of energy and the continuous energy flux through the system finally leads to a permanent rearrangement and reorganization of both the biochemical and the physical structure. Energy fluxes in soil are caused by the input of thermal energy at the soil surface, the precipitation of water, and the input of organic matter either through living plant roots or through plant residuals. Additionally, in agricultural systems, tillage and manuring is a significant input of energy.

The introduced energy is subjected to dissipation due to the general drive of the system toward thermodynamical equilibrium. Following the path of energy dissipation there are manifold structure-forming agents. First, the energy input itself is heterogeneous in space, mainly caused by the relief of the soil surface—also at a very small scale—and the structure of the vegetation cover. Second, the introduced energy provides the resources for a rich variety of soil biota and many organisms form their habitat according to their demands, once they arrived at a given location. Altogether, this leads to a highly heterogeneous spatial structure of material properties at a given time and a highly heterogeneous temporal structure of material properties at a given location.

There is a close relation between structure forming processes permanently fed by the persistent energy flux and the counteracting forces of structure decay, which finally stabilizes the system far from thermodynamic equilibrium with a highly heterogeneous structure. This phenomenon can also be observed in much simpler abiotic systems. Prominent examples are stable convection cells (Bénard convection) within a fluid that is permanently heated at one side as, for example, a pot of sauce on a hot plate. This type of self-organization leads to a “*dissipative structure*,” as introduced by Prigogine [24] where the ambition of energy dissipation is a constitutive element of structure formation. Another important ingredient of this type of self-organization is the nonlinear behavior of the system, which allows for a stabilization of the structure against external perturbations. The nonlinearity of impelling and depressant processes makes it possible that these counteracting processes balance out at a well-defined point, which therefore forms an attractor of the system. In permafrost soils we frequently find structures known as “sorted circles,” [11] which are quite similar to convection cells and the formation may be ascribed to similar processes.

Averaging over long time scales, however, may lead to the stable soil types and thus to the homogeneity implied by Jenny. The whole material of the topsoil may have passed the stomach of an earthworm within a few years [8]. In a forest there may be a tree at each location within some multiple of the trees lifetime. But the actual status of an ecosystem is heterogeneous and structured at any time and any spatial scale of observation and herewith provides a prerequisite for the observed biodiversity.

The small-scale heterogeneity of material properties and living conditions becomes manifest in several well-known phenomena of agricultural practice. For example, the burst in microbial activity after disruptions of the soil structure through, for example, tillage indicates that due to the small-scale structure, parts of the nutrient pool of the soil is protected from decay through physical separation of the organic matter from organisms. The subscale structure also allows the coexistence of aerobic and anaerobic zones within small distances, which results in a close side-by-side of completely different biochemical conditions. This may intensify the turnover of organic material, it certainly increases the diversity of the soil biota.

In the following, we intend to highlight the diversity of the physical structure, especially in soil, as a central part of agricultural production systems. We first give a short overview on different imaging techniques commonly used to gain insight to the various aspects of heterogeneous soil structures, and we present a collection of various examples for the spatial structure and organization at different spatial scales. We will highlight the related processes of structure formation as well as

the consequences for the actual material properties and functioning of the soil. Subsequently, we discuss the implications of the observed spatial heterogeneity and present various concepts for the organization of structure across spatial scales. Then, we propose some essential tools to describe complex spatial structures in a quantitative way as a prerequisite to account for spatial heterogeneity in science. These tools are generic in the sense that they can be applied to any structure irrespective of the spatial scale and irrespective of the specific instrument used to record the structure.

3.2 OBSERVABLE STRUCTURE AT DIFFERENT SCALES

3.2.1 IMAGING TECHNIQUES

Usually our eyes are the main instrument to explore the diversity of spatial structures. However, if we intend to compare, to communicate, or to quantify what is in front of our nose we have to refer to some sort of instrument that provides a quantitative signal in the form of an image. For instance, a digital camera provides a more quantitative version of our visual impression. Moreover, there are instruments that operate within different bands of electromagnetic waves, for example, radar, microwaves, or X-ray, and therefore, they are sensitive to other aspects of the object that are not detectable through visible light.

Generally speaking, a perfect instrument evaluates a function of the form

$$f(x) = i(x)r(x) \quad (3.1)$$

where $f(x)$ is the signal provided by the instrument at location x , which depends on the illumination of the object $i(x) \in [0, \infty]$, and the fraction of that illumination, which is reflected or transmitted by the object $r(x) \in [0, 1]$. Depending on the chosen quality of $i(x)$, we might obtain very different impressions of the same object since a specific $i(x)$ may highlight some specific aspects of the structure while others remain invisible.

Starting at a small scale, the structure of mineral grains can be recorded using a scanning electron microscope, where the material-specific emission of electrons is used to represent the object. At the scale of a few microns to millimeters, the investigation of thin sections using transmitted visible light provides information on the opacity, the refraction properties, or, if crossed polarized light is used, the birefringence of the constituent parts [5]. The color of the material becomes more clear when oblique incident light is used, which is also the typical illumination for the investigation of soil-polished blocks. An attempt to classify the vast diversity of structural features at the scale of thin sections and polished blocks was made by FitzPatrick [9].

At the scale of a soil sample of a few centimeters to some decimeters, the absorption of transmitted X-ray radiation provides information on the electron density of the material. This technique is especially attractive because it is a noninvasive method providing a full three-dimensional representation of the material. In soil, this technique is mainly used to determine the structure of bulk density and macropores [31], but also to quantify the spatial distribution of soil water [14]. At the scale of a soil profile up to the landscape scale, classical photography is typically used to record the visible structure. On the larger scales, larger wavelengths can be used to record structural properties as exploited by different geophysical tools, (e.g., microwaves and georadar) [25]. Finally, some instruments can be mounted on aeroplanes or satellites to obtain an overview on global structures, which is typically referred to as remote sensing.

An important aspect that is obvious from the general equation (3.1) is that we might improve the quality of the obtained images by optimizing the reflective or transmissive properties of the object $r(x)$. This is done, for example, by adding dye to the resin of impregnated samples to improve the contrast between pores and solid material [20] or sophisticated staining techniques were used to visualize the distribution of root and microorganisms in soil [1]. In field experiments the

preferential flow paths of water can be marked using dye tracers [10]. The same is possible for soil columns using heavy ions detectable with X-ray tomography [22].

All available imaging techniques are applicable to a limited range of spatial scales and provide information at a limited resolution. In figure 3.1 these characteristics are summarized and related to the characteristic size of various earth burrowing organisms.

3.2.2 THE EVIDENCE OF STRUCTURE AT ANY SCALE

In the following we present some typical examples of structure in soil, starting at the microscopic level of a few millimeters and moving up to a macroscopic level of several kilometers. Related to the various structures, a short interpretation with respect to the generation processes and with respect to their impact on actual processes and material properties are given. Thereby, the close interrelation of structure, function, and structure-forming processes as well as their contribution to the diversity of the system becomes evident.

Example 1 (fig. 3.2). The separation of soil constituents due to their specific potential for translocation is one of the most important abiotic structure-forming processes in soil. An illustrative and well-understood example is the vertical translocation of clay within macropores into deeper soil horizons, where the clay is deposited at the pore walls. There, the resulting clay coatings separate completely different subspaces: the compact matrix essentially is made up of quartz grains (silt size) with some iron oxide covers and opaque particles, which are iron oxides and highly humified organic matter. The largest root pore in the figure contains residues of two smaller roots.

This feature is also related to actual processes. It demonstrates that the root channels serve as preferential flow paths through which the vertical transport of solutes to deeper soil horizons is facilitated. Beside water and solutes, soil aeration is expected to take the same path. Altogether, this may also influence the chemical conditions in the vicinity of such preferential flow paths. An indication of this phenomena can be seen in figure 3.2, where the enrichment of opaque iron oxides close to the large root channel is evident.

Example 2 (fig. 3.3). The activity of the meso and macro fauna in soil, especially earthworms, enchytraids, and collembola but also larger animals such as hamsters and moles, are a significant source of the diversity of living conditions in soil. The bioturbation as a consequence of the feeding and burrowing activities may lead to a homogenization of the material at locations where the activity is high. At the limits toward unfavorable living conditions, however, the movement of animals and their activities in transporting materials may lead to a high contrast of material properties. In the subsoil shown in figure 3.3, the rounded, dark aggregate is a cast of an earthworm containing organic

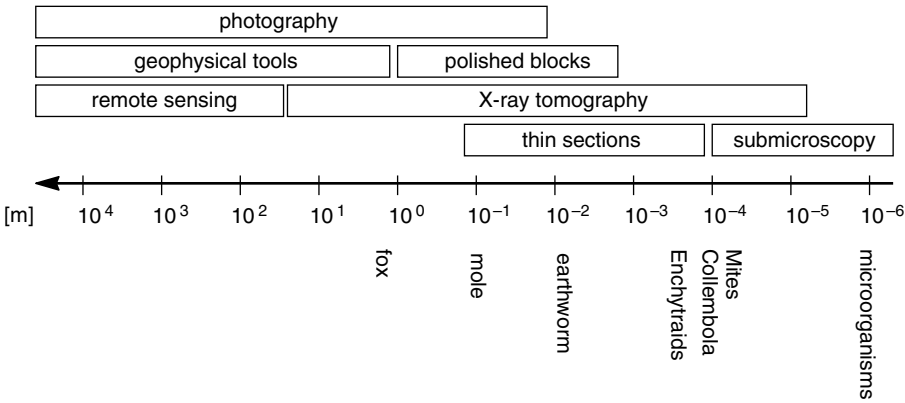


FIGURE 3.1 Typical instruments for the investigation of soil structure, the related scale of observation, and the typical body size of different earth-burrowing animals. Note that the size of the habitat of the species is typically by a factor of 10^2 to 10^3 larger than the body size and the resolution of the various instruments (pixel size) is by a factor of 10^2 to 10^3 smaller than the scale of observation.

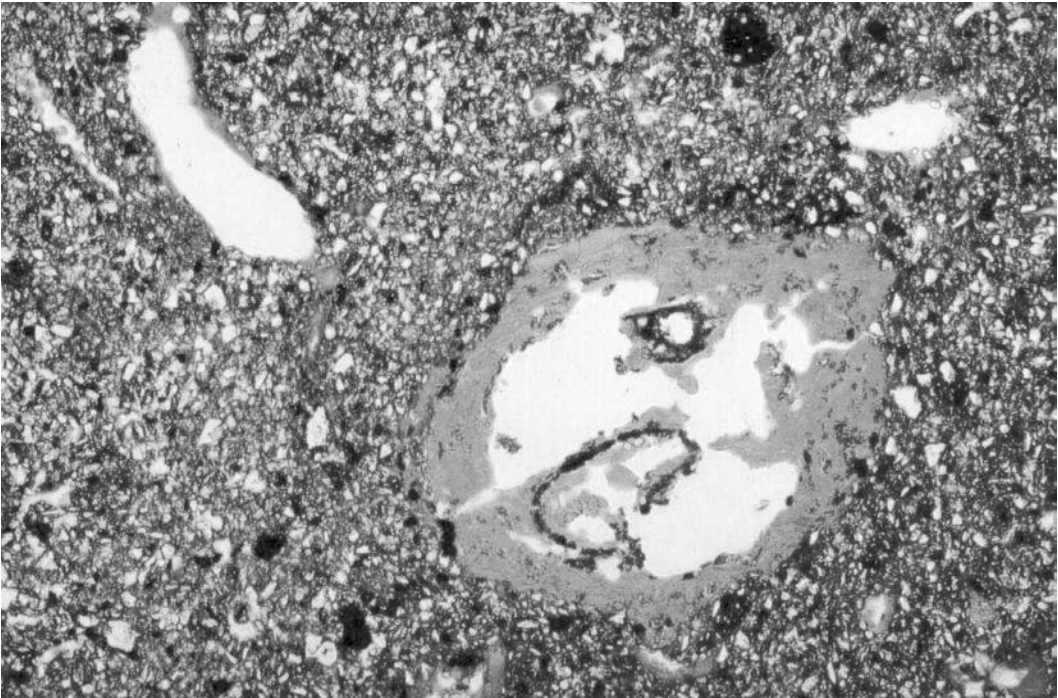


FIGURE 3.2 (See color insert following page 170) Orthic Luvisol from Loess, BtC-horizon, depth 90 cm, thin section, bright field, natural width 3.6 mm.

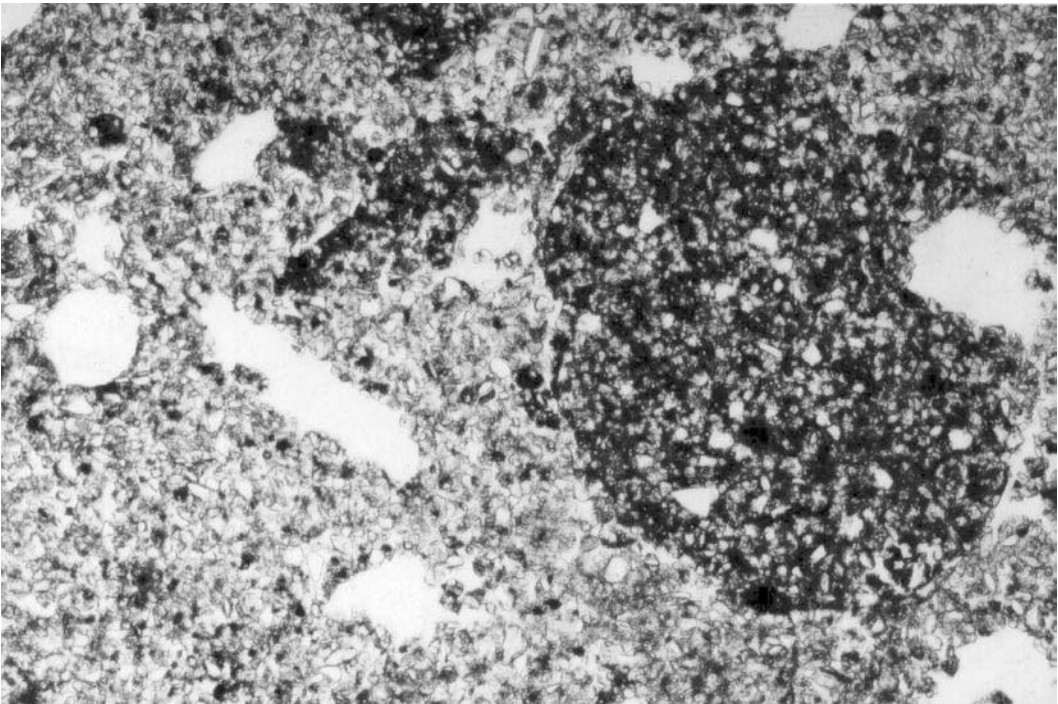


FIGURE 3.3 (See color insert) Tschernosem, AhC-horizon, depth 50 cm, thin section, bright field, natural width 3.6 mm.

material from the top soil. The local enrichment of organic material obviously attracted enchytraids. This can be concluded from the cylindrical channels with a typical diameter of some 0.3 mm. Herewith, the earthworm cast initiated the generation of a subspace with contrasting physico-chemical properties characterized by an increased volume of macro pores and the presence of various organic substances. Eventually, this micro fabric is colonized by an adapted population of microorganisms. Such structural features are continuously generated. Once created, they slowly disappear with the gradual decomposition of the organic material. Finally, this leads to an accumulation of highly resistant organic compounds, as visible by the homogeneously distributed opaque organic particles within the soil matrix.

Example 3. Figure 3.4 is taken from the same soil as figure 3.3, but closer to the soil surface. In this depth the activity of earthworms is much higher compared to the subsoil, which is evidenced from the rounded bow structure of both the voids and the arrangement of the various components inside the soil matrix. Obviously the structure of the whole material is the result of a high activity of earthworms so that homogenization of the material properties could be expected. Actually, an intense mixture of organo-mineral material (reddish, brownish) with the mineral grains can be found. However, inside the soil matrix there are zones showing a considerable enrichment of organo-mineral material. This segregation of different constituents is a result of the kneading of the highly wetted material in the gut of the earthworm. This leads to considerable variability of material properties at a scale of some 100 μm with consequences for the hydraulic properties as well as for the mechanical stability of the resulting aggregates and for the quality of the material as substrate for microorganisms.

Example 4. Figure 3.5 shows again the same soil as figure 3.3 and figure 3.4, but at a larger scale and in depth between the previous figures. The fabric is sharply separated into a coherent matrix and a crumbly structure of fresh earthworm casts. Note that the separation of the material inside the casts, as shown in figure 3.4, is still visible at this scale. The loose packing of aggregates

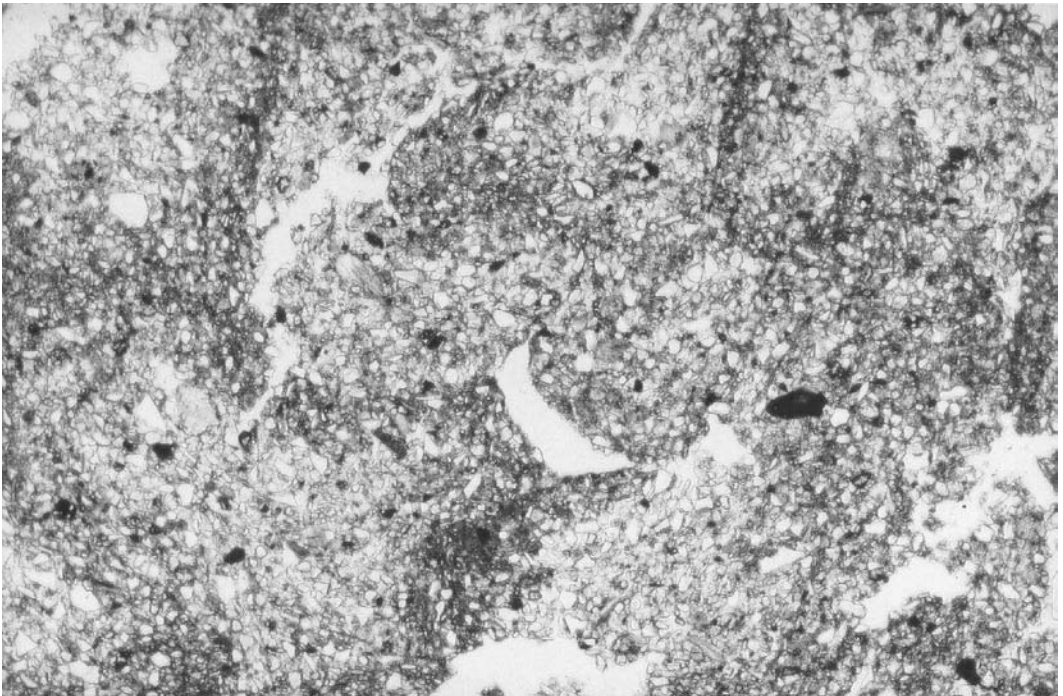


FIGURE 3.4 (See color insert) Tschernosem, Ap-horizon, depth 8 cm, thin section, bright field, natural width 3.6 mm.

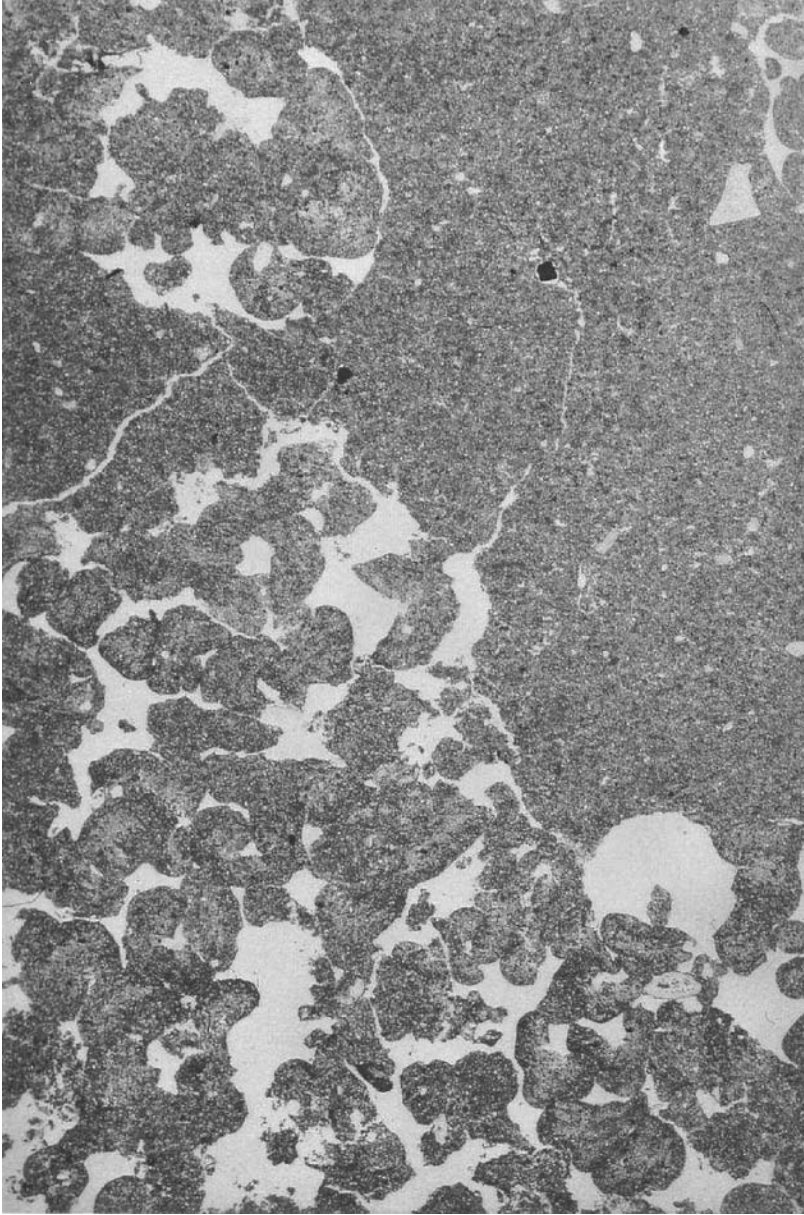


FIGURE 3.5 (See color insert) Tschernosem, Ap-horizon, depth 40 cm, thin section, bright field, natural width 30 mm.

forms a network of continuous macropores, which may serve as a living space for species that are not able to burrow by themselves, as, for example, some Chilopoda or Gamaside mites, which are common predators in soil. This illustrates the microscopic diversity of habitats just based on the available pore diameters separating the potential residents according to their body size. Moreover, the physical properties of the crumbly fabric are characterized by both a high water capacity inside the aggregates and a permanent availability of oxygen.

Example 5. The soil matrix shown in figure 3.6 is speckled by iron oxides (reddish spots) and root channels (small, rounded black dots). On a larger scale, this homogeneous matrix is separated by cracks due to the swell-shrink dynamics of the material having a clay content of about 50%.

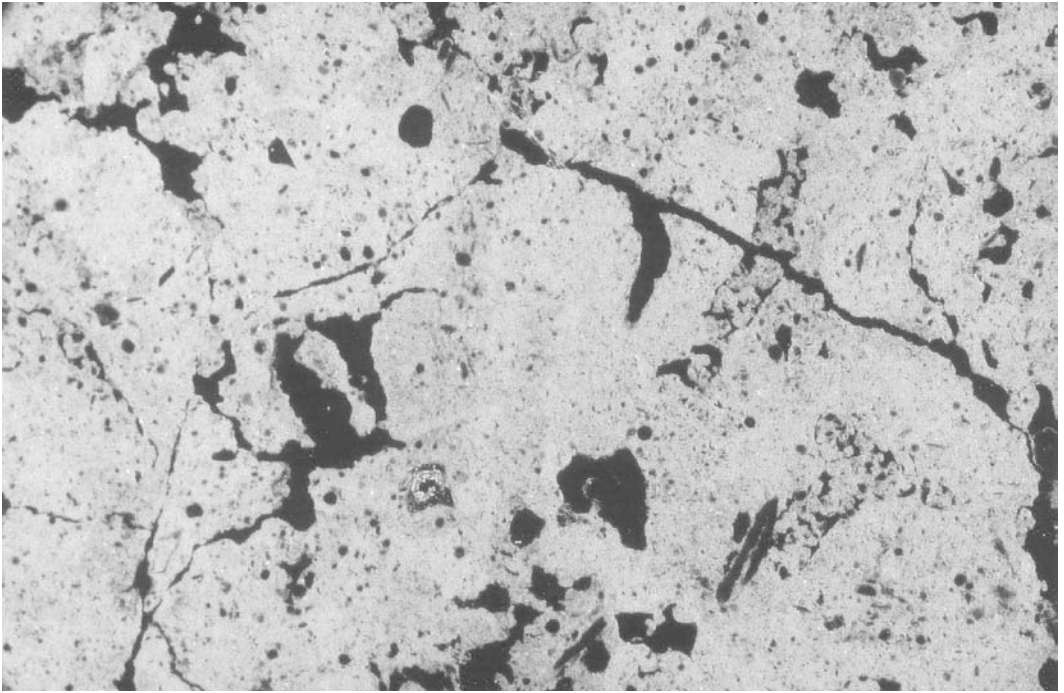


FIGURE 3.6 (See color insert) Terra Fusca, AhBv-horizon, depth 3 cm, thin section, dark field, natural width 9 mm.

The dominant structural feature consists of macropores showing a multitude of irregular forms. However, all these macropores probably originate from the same source, which is the burrowing activity of earthworm species. The formerly cylindrical burrows are altered because they are suitable habitats for smaller organisms as enchytraids, collembola, or small earthworms, all of them animals with high soil-feeding activity.

This structure illustrates the continuous formation and alteration of structure in soil, which finally leads to a smooth transition between different structural features at a given time and a given spacial coordinate.

Example 6. Figure 3.7 shows examples for the application of X-ray tomography to visualize the structure of macro pores and of bulk density at a scale of some centimeters. The sample is taken from the ploughed horizon of a conventionally tilled arable soil. The image on the left shows a horizontal section through the cylindrical sample. The gray values correspond to the local X-ray absorption and herewith are related to the local electron density. Stones of high density (white) can be clearly distinguished from macropores (black). The structure of dense aggregates (light gray) lying in a more loose matrix (dark gray) is evident and is the result of the farmers' activity.

The three-dimensional structure of macropores can be reconstructed (right image) by this nondestructive imaging technique. The observed high connectivity of the network of macropores is crucial for the hydraulic properties of the material, especially the saturated hydraulic conductivity and the supply of oxygen to deeper soil horizons. This connectivity is periodically disturbed by tillage practices but is typically restored by the biological activity (i.e., roots and earthworms).

Example 7. An initial stage of soil formation is shown in figure 3.8. The physical weathering of the triassic marl clay, mainly crack formation, allows the penetration of roots following the crack pattern. The resulting enrichment of organic material along the root paths forms the substrate for heterotroph micro and meso fauna decomposing the organic residues. This, in turn, improves the conditions for plant roots, which finally leads to a self-accelerated process of soil formation. Here,

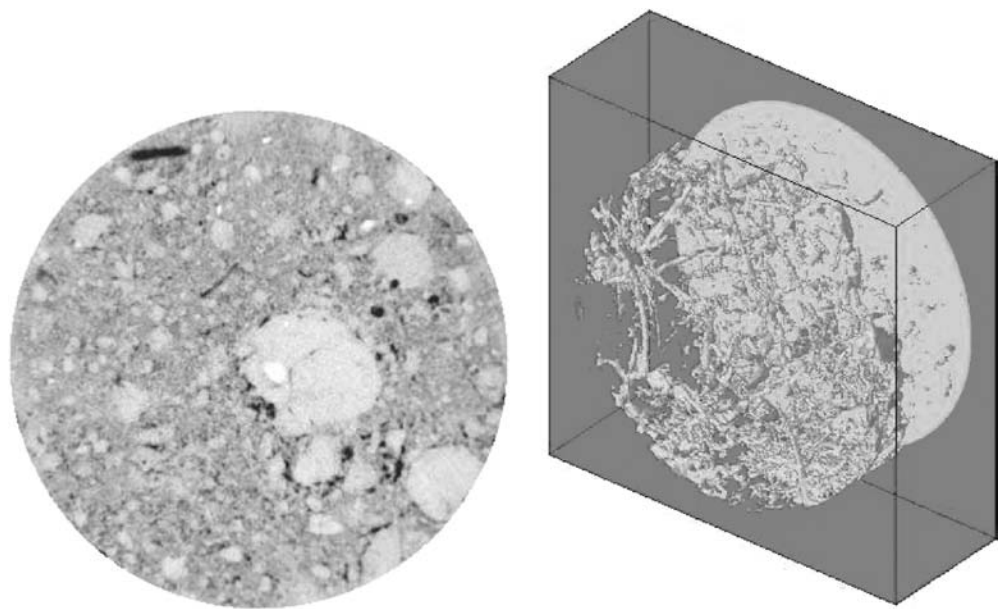


FIGURE 3.7 Haplic Calcisol, Ap-horizon, depth 2-12 cm, X-ray tomography, natural width 162 mm.



FIGURE 3.8 (See color insert) Triassic marl clay, Cv-horizon, depth 60 cm, natural width 10 cm.

this process is limited by the slow root production within the considered depth of the profile, which stabilizes the structure in the actual state. The resulting structure is composed of two materials with a high contrast in physical and chemical properties, which are clearly separated by a sharp interface.

Example 8. Figure 3.9 shows the total soil profile of figure 3.8. Four different horizons are clearly separated by relatively sharp boundaries. The first boundary is sharp because of the absence of large burrowing earthworm species or other animals who are capable to bring the organic matter produced at the soil surface deeper into the soil profile. The second (somewhat above the knife) is a boundary between different materials as a result of solifluction (i.e., silty, weathered loess over clayey marl). The third one (below the knife) is one of the chemical weathering of the clayey marl.

Example 9. Figure 3.10 shows the profile of a 80-cm deep loamy-clay arable soil on a calcareous plateau (Villamblain, France). It was cultivated with minimum tillage the last 5 years. Three different horizons can be distinguished: (1) the first 10 cm with a loose crumbly structure due to tillage; (2) the old ploughed horizon, with a ploughpan at the lower limit at 30 cm depth; and (3) the subsoil below. The dark green areas are stained by a dye, which was infiltrated at a constant flux of 13 mm/h.



FIGURE 3.9 (See color insert) Solifluction cover (clayey silt) on weathered marl clay, natural height 0.7 m.

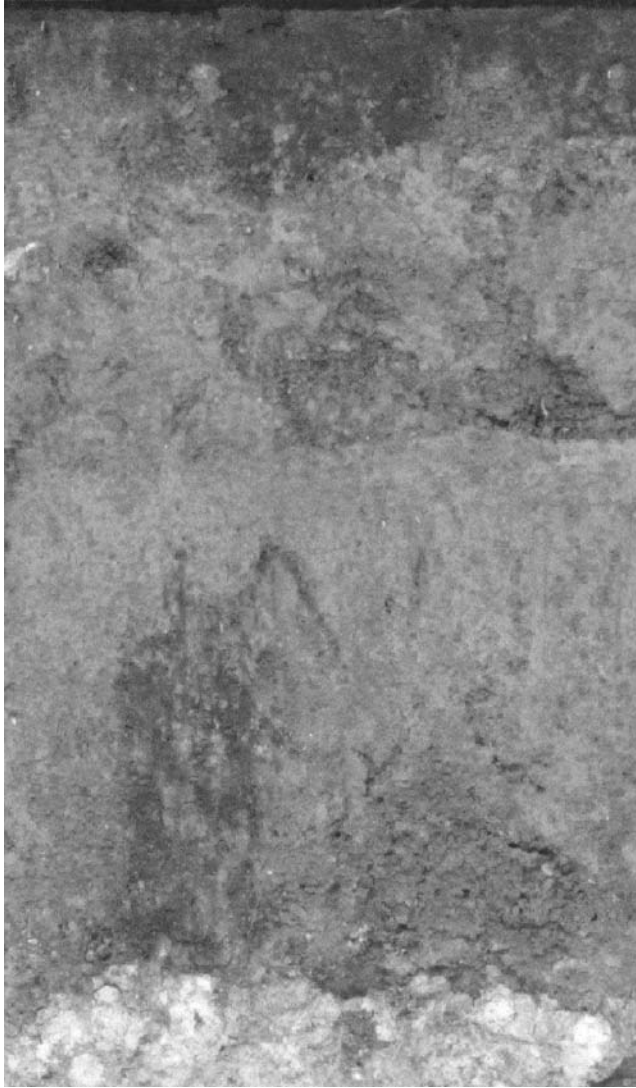


FIGURE 3.10 (See color insert) Haplic Calcisol, France, natural width 0.4 m.

This image demonstrates the effect of structural diversity rather than the structure itself, which is not clearly visible at this scale. Actually, the old ploughed horizon was compacted during the period of reduced tillage due to surface traffic and only a few continuous macropores formed by roots and/or earthworms serve as preferential flow paths toward the subsoil. As a consequence, (1) vertical transport is much faster as would be expected from mean hydraulic properties and (2) a considerable fraction of the soil is shaded from the input of water and solutes.

Example 10. Typically, the structure of the above-ground vegetation has a counterpart in the subsurface, as illustrated by the root system of an oak tree in figure 3.11. The homogeneous soil profile shown in figure 3.9 originates from the same site but its location is well in between the trees. The spatial distribution of roots is clearly related to the distribution of the plants. Especially in forests, this introduces a large scale component to the soil structure. Actually, roots exhibit considerable structure-forming potential not only through the penetration of the soil matrix but also through the local energy input via organic substances and the subsequent decomposition along a specific food chain. Moreover, the overground structure of the crowns induces a specific pattern of



FIGURE 3.11 Solifluction cover on weathered marl clay, natural width 4 m.

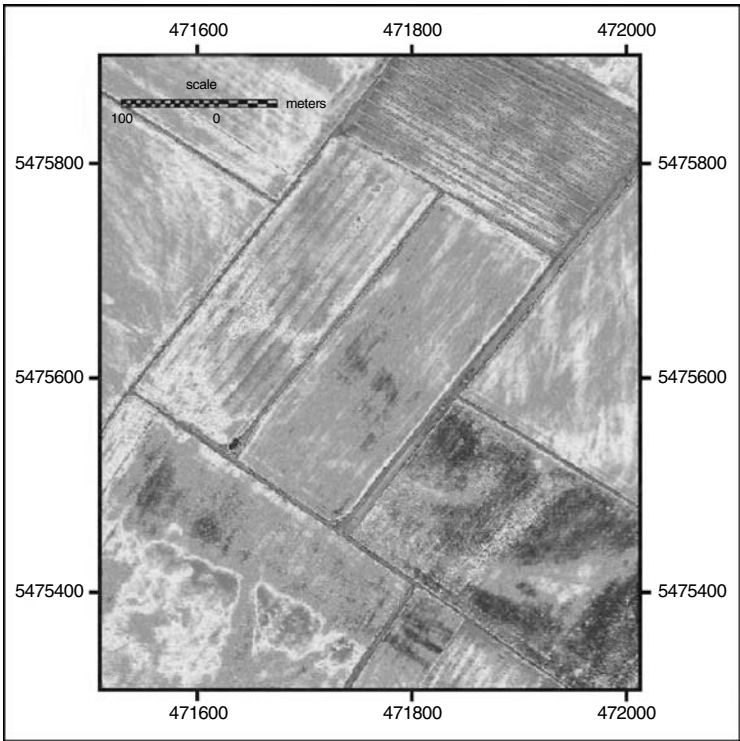


FIGURE 3.12 (See color insert) Air photograph of surface temperature, Rhein valley, Heidelberg, natural width 0.5 km.

precipitation caused by interception. This pattern depends on the morphology of the crown and the bark and is a source of heterogeneity also for soil properties.

Example 11. Beyond the scale of single soil profiles the diversity of soil properties can be explored by remote sensing techniques. Figure 3.12 shows a photograph for the surface temperature of arable land taken from an aircraft in summer. At this time the maturity of the cultivated crops reflect some soil properties, especially the availability of water. Some of the features are clearly related to the specific crops and the specific tillage practices on the individual plots. Others, however, are continuous even across different fields and reflect the subsurface structure. In this case it is a change of loamy-clay sediments from the Neckar River and coarse-grained sediments from the Rhein River with a diagonal orientation from the lower right corner upwards to the left visible in the lower half of the image. This approach was recently demonstrated by Sommer et al. [27] who also provided the image in figure 3.12.

Example 12. Figure 3.13 is obtained from Landsat satellite data after supervised segmentation of the image into different types of vegetation [17]: forest (black), savanna (grey), and crops (light grey). The image was taken in 1975 and the observed distribution mainly reflects the relief of the catchment with a small stream running from north to south. Agriculture is concentrated at the more

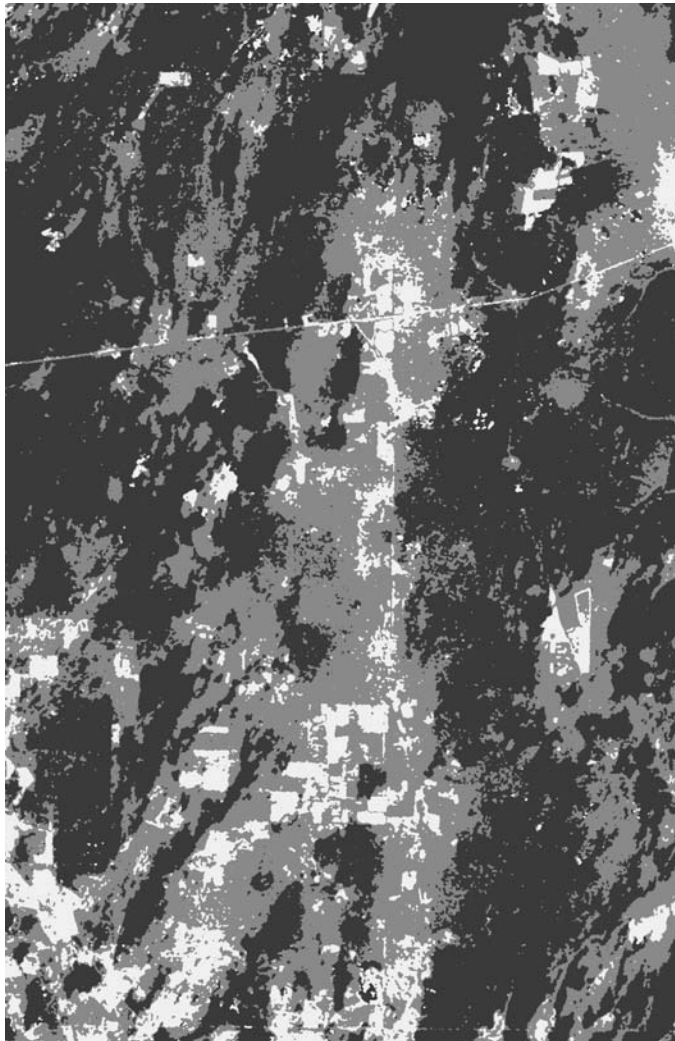


FIGURE 3.13 Landsat satellite image, Chaco, Argentina, natural width 37km.

fertile locations along the valley. Repeated recordings allow for a detailed analysis of the temporal change of land-use development and diversity (see below).

For most organisms the spatial distribution of environmental properties including the connectivity and distance distributions is a crucial criteria for the quality of their habitat. Also with respect to soil degradation and surface hydrology, the spatial pattern of the protective natural vegetation on one hand and of intense agricultural management on the other hand is critical.

3.3 CONCEPTS OF HIERARCHICAL ORGANIZATION OF STRUCTURE ACROSS SCALES

There are different approaches to conceptualize the fact that our natural environment including soils and landscapes is typically structured at any scale. Such concepts are highly required to characterize the diversity of soil structure and to transfer information from a small scale to the next larger scale. The latter is a notorious problem for many practical aspects in soil science where the results of some measurements obtained for samples of a given size have to be extrapolated to other locations and other spatial scales.

Before going into details of the different conceptual approaches, it is necessary to define some related terms, which are indispensable to get a clear idea about the hierarchical organization across spatial scales.

3.3.1 OBSERVATION SCALE AND RESOLUTION OF THE INSTRUMENT

There are two basic measures that are directly related to the instrument used to observe an object, irrespective of the used instrument and irrespective of the spatial scale: The first is the field of view of the instrument, which we will refer to as the *observation scale* Ω . The second is the size of the integration volume of the measurement inside this field of view, which simply is the pixel size ω . Although our object is typically three-dimensional, the measurements are often taken from a two-dimensional or even one-dimensional cutout. Hence, the dimension of Ω and ω varies correspondingly. For convenience we will in any case express Ω and ω in units of a length. Comparing the extension of the field of view in the different spatial directions, we choose the smallest extend for Ω because this measure provides the limit over which the structure can be observed continuously. In contrast, we choose the largest extend for ω because this provides the limit of resolution below which the structural components cannot be detected anymore. Most of the imaging techniques operate with isotropic pixels within isotropic windows so that this choice is of no importance.

The imaging techniques used here yield a continuous measure, meaning that the field of view is completely covered by pixels, that is, $(\Omega/\omega)^d$ is the total number of pixels in an isotropic d -dimensional image. This is different to other measurement techniques frequently applied in soil science, where only a sparse sampling of Ω is possible, for example, when measuring hydraulic or other properties for a number of samples within a plot. Then, the resolution ω within the plot of size Ω is given by the distance of the samples and the integration volume of the single samples is an additional characteristic measure called *support scale* of the measurement in hydrology [4]. In that case the continuous structure within the plot has to be inferred from the sparse measurement, which demands some sort of interpolation. This invokes the field of geostatistics [6].

3.3.2 MINIMUM CHARACTERISTIC LENGTH AND REV-LENGTH OF THE STRUCTURE

Given the representation of an object at a certain observation scale Ω and resolution ω it is typically possible to distinguish different entities based on their specific attributes, meaning we observe some kind of structure. In the following such entities are termed *structural units*. In figure 3.14, several structural units are distinguished: macropores, root channels (macropores having a specific form), cast infillings, and the porous soil matrix. Note that a structural unit may be separated into several

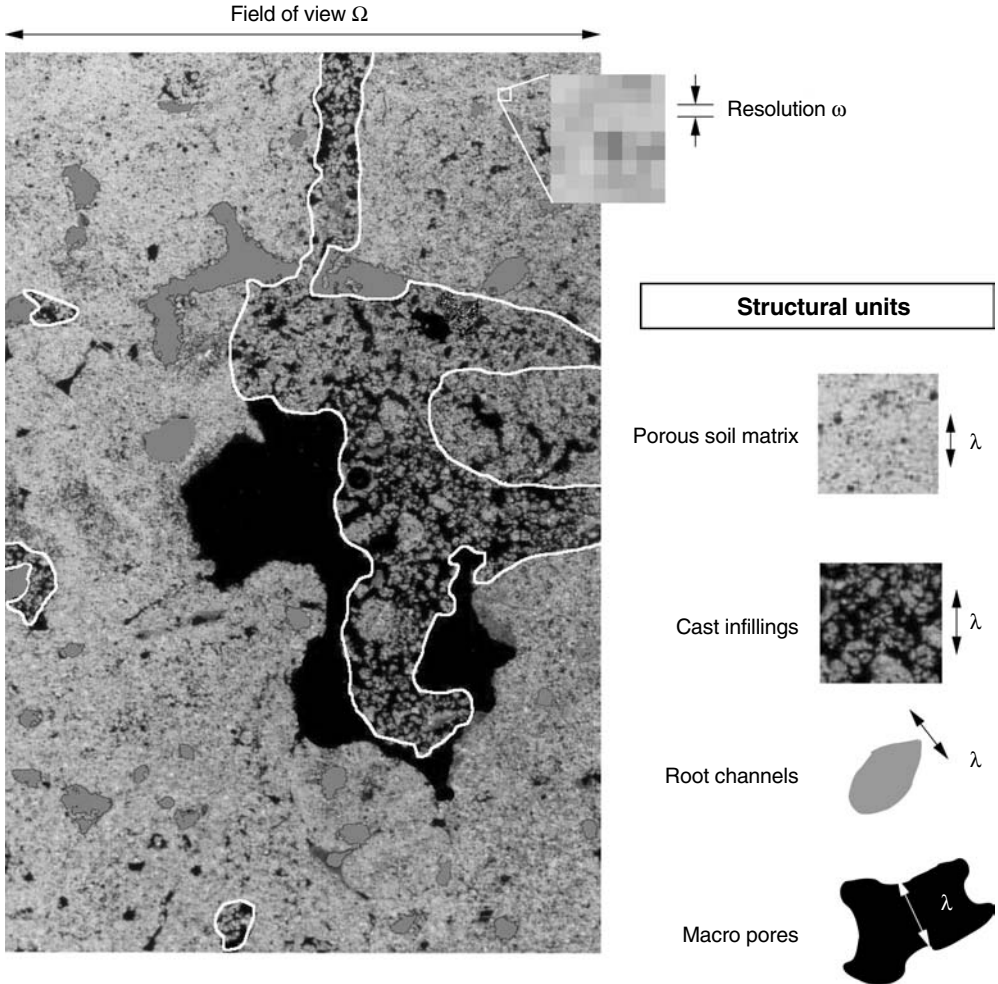


FIGURE 3.14 (See color insert) Vertical section through the subsoil of a Tschernosem (same profile as figure 3.3, figure 3.4, and figure 3.5, C-horizon, thin section, incident light, depth 70 cm, natural height 13.5 mm). Observation scale Ω and resolution ω are related to the instrument used to record the image. The different structural units are distinguished by a specific texture, as indicated in the right column. They have a specific minimum characteristic length λ . The REV-length Λ of the structural units cannot be shown since $\Lambda > \Omega$. (See text for a detailed explanation.)

isolated parts. This, however, might be an artifact due to the two-dimensional sampling of a three-dimensional object. For instance, the isolated root channels in the two-dimensional section shown in figure 3.14 might be perfectly connected in three dimensions.

Related to each structural unit there are two characteristic lengths that can be defined irrespective of the type of structural unit and irrespective of the spatial scale: the maximum characteristic length, which is considered to be the *REV-length* Λ of the structural unit, and the *minimum characteristic length* λ of the structural unit. The REV-length Λ can be interpreted as the minimum side length of a cubic sample that would be required to capture the structural unit representatively. This definition corresponds to the well-known concept of a representative elementary volume (REV), which can also be defined for various material properties [3]. Here we only consider the geometry of the structural units and the REV-length is reached as soon as the mean value of the geometric properties becomes invariant under translation of Ω . The minimum characteristic length λ is the minimum size of a cubic

sample that would be required to identify the structural unit as such. For the example in figure 3.14 the REV-length Λ of the root channels is expected to be somewhat larger than the size of the image while the minimum characteristic length λ is the typical diameter of individual channels. As for the instrument-related characteristic lengths, we measure Λ and λ in units of length.

3.3.3 STRUCTURE AND TEXTURE

In the following we use the terms *structure* and *texture* as scale-independent attributes of heterogeneous materials and we use the characteristic length scales introduced above to separate structure from texture.

In summary, *structure* at a given scale is the spatial arrangement of the various structural units, which themselves are identified by their specific *texture*. Moreover, when going from small scales to larger scales, structural units turn into texture at some point while new structural units may appear. This has also some important implications for the upscaling of material properties as, for example, water retention, hydraulic, or thermal conductivity. Assuming that material properties reflect the geometric form of the materials' constitutive components, we would expect that the transition from structure to texture corresponds to the point where a heterogeneous material turns into a macroscopically homogeneous material, that is, that the microscopic heterogeneity can be replaced by an averaged effective description as discussed further below.

Given a representation of an object as, for example, the thin section in figure 3.14, we identify different structural units u if their REV-length appears to be larger than the observation scale, that is, $\Lambda_u > \Omega$. In figure 3.14 this is definitely true for the "macropores" and the "cast infillings," which therefore have to be considered as structural units. Consequently, this has to be also true for the "porous matrix," which constitutes the embedding material of the structural units. It is less obvious for the unit of "root channels" because this structural unit might be captured representatively by the image, that is, $\Lambda_{\text{rootchannels}} < \Omega$. In this case, the structural unit becomes part of the *texture* of the embedding material. Actually, the image in figure 3.14 is at the limit $\Lambda_{\text{rootchannels}} \approx \Omega$ where the structural unit "root channels" turns into a textural component of the structural unit "porous matrix."

We use the terms structure and texture in a broader sense compared to the classical meaning in soil sciences, where texture is related to the size distribution of primary particles of the soil and structure is mainly related to the organization of these particles in form of aggregates. However, this specific meaning is included in our more general definition since aggregates can be considered as the next higher level of the organization of primary particles.

The transition from structure to texture when moving from smaller to larger scales is illustrated in figure 3.15. Thereby, the overall structure is hypothetical: at the scale of the soil profile we find two horizons and the structural unit of macropores that spans over both horizons. Moving toward smaller scales within horizon A, we first identify cast infillings and later root channels as additional structural units. Note that "moving toward smaller scales" means decreasing Ω and ω while Ω/ω remains constant. With decreasing scale the units containing solid phases break down in additional subunits. In contrast, the various classes of pores always have a lower size limit.

The vertical extend of the boxes in figure 3.15 reflects the specific section of scale over which the structural unit exists. It can be associated with the dimensionless quantity $\Gamma = \Lambda/\lambda$ and depends on the anisotropy and the spatial distribution of the structural unit. Large values of Γ as for the macropores in our example lead to practical problems since the instrument required to detect that type of structure should meet the condition $\Omega/\omega > \Gamma$. Note that the REV-length Λ of constitutive elements of a large-scale structural unit equals the characteristic length λ of the structural unit buildup by these elements.

The hierarchical structure as indicated in figure 3.15 is typical for soils. In the preceding section only a few illustrative examples could be presented. Though extremely beneficial regarding biodiversity, this type of heterogeneity causes serious difficulties for the direct measurement of soil properties: the results generally vary in space at any scale and they generally depend on the size

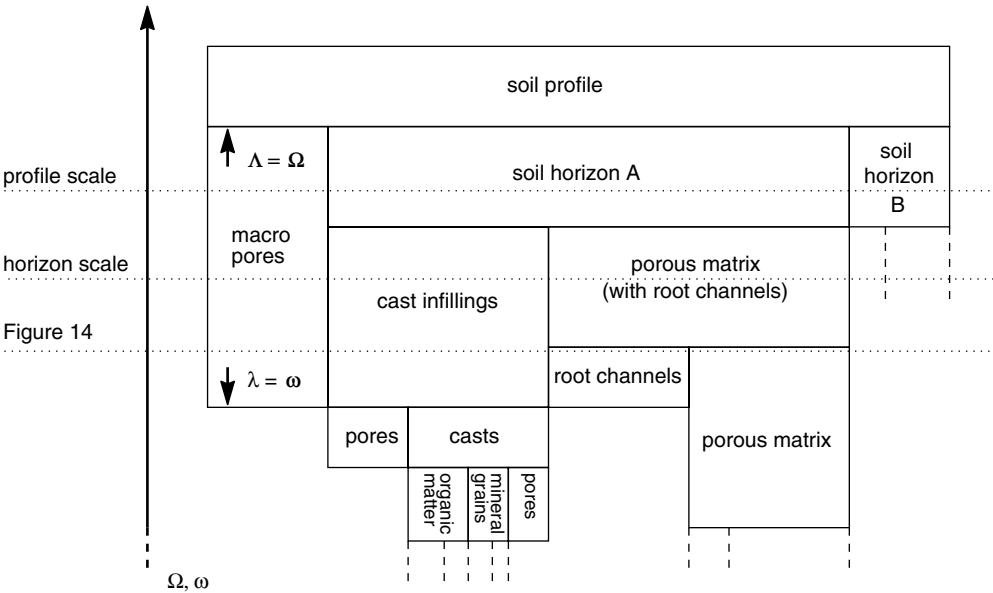


FIGURE 3.15 Schematic sketch of structural units at different scales following the example shown in figure 3.14. The scale of this figure is indicated by the dotted line. Each structural unit is represented by a rectangular box. The vertical extend of each box corresponds to the “scale window” where the structural unit exists. The limits of the scale window are defined as indicated for the macro pores. Note that different structural units merge at different points to build up the texture of a new structural unit at the larger scale.

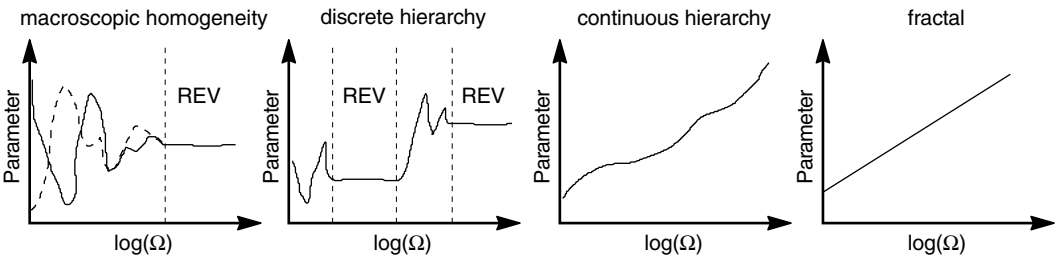


FIGURE 3.16 Characteristic evolution of some measured parameter as a function of the observation scale Ω for different concepts of structural organization. Note that the parameter value might also decrease with increasing observation scale.

of the sample. During the 1970s this fact entered the laboratories of soil science as the ghost of spatial variability and the failure of model predictions was frequently ascribed to this omnipresent phenomenon. In the following different concepts to formalize the spatial diversity of structure in soil are discussed that may help to cope with spatial heterogeneity.

3.3.4 MACROSCOPIC HOMOGENEITY

The most classical approach is the concept of macroscopic homogeneity, assuming that an overall REV does exist. In the framework of our characteristic measures, this implies that beyond some critical scale Ω_c the material is not structured anymore and all the small-scale structural units i have turned into texture, that is, $\Lambda_i < \Omega_c$. Hence, any measure taken at this scale yields a stable value in space and time and all the subscale heterogeneity can be averaged to produce some “effective” values. This is illustrated in figure 3.16 (left graph). Prominent examples are bulk density,

water retention characteristics, or hydraulic conductivity, which are effective properties averaged over the complex porous structure at the subscale. However, due to the multiscale diversity of soil structure, such effective properties are only valid for a rather narrow window of spatial scales or they do not even exist.

3.3.5 DISCRETE AND CONTINUOUS HIERARCHY

The assumption that we find structure at any scale and the postulation that the characteristic lengths defined above do exist for the various structural units leads to the idea of soil as a discrete hierarchical system, as proposed by Cushman [7]. Going from the small scale to the large scale, that is, increasing Ω , the REV-length Λ_i of the actual structure is reached at some point where the structure turns into texture and we may discover the properties of macroscopic homogeneity. When increasing Ω further, however, a new structural unit with $\Lambda_{i+1} > \Lambda_i$ appears so that we are back to a heterogeneous structured material, which becomes homogeneous at a larger scale, and so forth (fig. 3.16). Hence, for a discrete hierarchical material, the notion of a REV is merely a local and not a global property. Such a discrete hierarchical organization reflects the idea of the diversity of structure-forming processes each having a typical length scale. This concept is implicit in many approaches in geosciences where soil aggregates, soil horizons, or geological facies are considered to be distinct units.

The prerequisite for a discrete hierarchy is that the characteristic scales of the different structural units, Λ_i , can be clearly separated. This is possible if $\Lambda_i \ll \Lambda_{i+1}$ and $\lambda_{i+1} > \Lambda_i$, which, for example, is the case for the soil profile in figure 3.9. Here, the structural units inside the soil horizons are much smaller than the thickness of the horizons. Another example of discrete hierarchy from a smaller scale is given by figure 3.5, where the characteristic scale of the structure inside the earthworm casts or inside the coherent soil matrix is clearly separated from the large-scale structure built up by earthworm casts and the coherent matrix.

In contrast to such a discrete hierarchy, the characteristic scales of various structural units might be interlaced and nested. Then, although the structure is still hierarchical, the different hierarchical levels cannot be separated anymore. This leads to the notion of continuous hierarchy [7] where no local REV's can be found (fig. 3.16). A practical consequence of continuous hierarchy is that the material properties change continuously with the observation scale Ω . An example of this type of organization is given by figure 3.14 where the "scale window" of the different structural units, that is, root channels, cast infillings, and macropores, overlap, which is also illustrated in figure 3.15.

3.3.6 FRACTAL STRUCTURES

Another concept was developed by Mandelbrot [18] who realized that some natural objects may exhibit self-similar structures or heterogeneities, at any scale of observation. This led to the notion of fractals, which negates the existence of stationary material properties and postulates the self-similarity of structure across spatial scales. It can be considered as a special case of continuous hierarchy where a power-law relationship between the observation scale Ω and some material properties ϕ can be found. This is an attractive approach since all the multiscale heterogeneities can be lumped into a single value, the fractal dimension, which can be obtained from the slope of a log-log plot of $\phi(\Omega)$ (fig. 3.16). For a review of the application of fractal geometry to soil structure, see Baveye and Boast [2]. However, it is not self-evident that soil should exhibit fractal properties. The self-similarity of the structure would suggest some self-similarity in the generation processes of the structure. In soil, however, the structure-generating processes are manifold and quite different at different scales, for example, packing of soil particles, the formation of soil aggregates due to swelling and shrinkage, the generation of macropores by animals, the formation of soil horizons and patterns of soil types due to the parent material and soil genesis.

3.4 QUANTIFICATION OF STRUCTURE AND DIVERSITY

3.4.1 GENERAL REMARKS ON IMAGE ANALYSIS

To get a handle on the structure and its diversity at a given spatial scale a quantitative characterization of the spatial structure is required. In the following it is supposed that the relevant structural units can be clearly detected and separated through a suitable instrument, eventually followed by a suitable procedure of data processing. Thereby, when analyzing natural systems, the major difficulty is related to the fact that the structure-forming processes typically come along with counteracting processes of structure decay. This inevitably lead to a somewhat fuzzy transition between different structural units, which may cause difficulties in separating structural units. One example is given in figure 3.6. However, the introduced fuzziness is typically small.

The broad field of image processing dealing with a large variety of methods to improve the quality of images in order to identify the relevant structure is not discussed here and the reader is referred to comprehensive textbooks [12]. In contrast, we focus on various approaches to analyze the observed structure toward a quantitative characterization of morphology and diversity.

The fast development of quantitative image analysis was an accompaniment of the exploding development of computer technology during the last three decades. Today there exists a huge variety of possible measures that can be efficiently calculated for digital representations of natural structures. In soil science, digital image analysis was initiated by Jongerius et al. [16] using first-generation hardware for digital image recording, the Quantimet. For the complex structure of pore space in soil, Murphy et al. [21] introduced a collection of measures to quantify shape and size of structural features. Since then, besides the enormous increase of computing power, also the theoretical fundament of image analysis was expanded including stereology [30], the set theoretical approach of mathematical morphology [26], and its application for digital image analysis [28].

In the following we concentrate on a small but significant set of measures that have some important properties:

- They are unambiguously defined
- They can be determined easily
- They have a clear stereological meaning (i.e., there is a clear relation to other measures obtainable from subsamples of lower dimensions)
- They are independent of the spatial scale

These measures provide an extensive description of the mean properties of a structural unit. However, as is shown below, the specific spatial distribution as well as the specific distribution of size, shape, and topology are lost through averaging. Therefore, the basic measures are extended using the concept of mathematical morphology to get a more comprehensive description.

3.4.2 BASIC MEASURES (MINKOWSKI NUMBERS)

Evidently, the mere number of different structural units is a first simple measure characterizing diversity. Moreover, the morphological form of the individual structural units is another significant property with regard to their quality as living space. The morphological form includes the size, shape, spatial distribution, and topology of the structural unit. The topology describes the spatial connectivity and herewith it is a highly relevant property not only for physical processes. For most organisms, the quality of habitats critically depends on the question of whether there is a continuous path from one place to another or not.

We consider a structural unit X , which is not known completely but only inside a certain field of view Ω^d where d is the spatial dimension. For most structural units in nature we find $d = 3$ but often, for practical aspects of sampling strategies, only some cutouts of lower dimension are

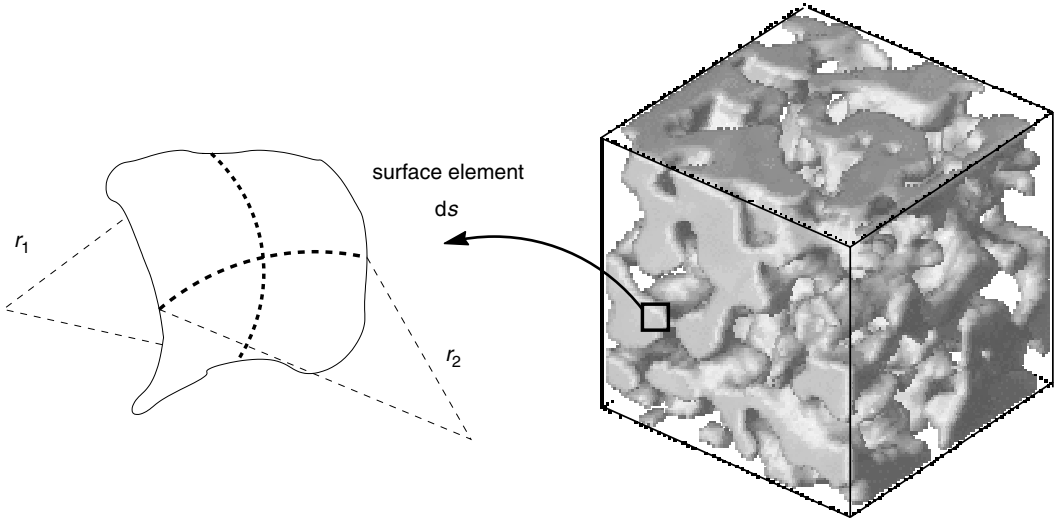


FIGURE 3.17 A structural object is unequivocally defined by its boundary. At each location on this boundary the principle radii of curvature (minimum and maximum) r_1 and r_2 are defined. The Minkowski numbers are obtained as integrals over the surface elements ds .

available, for example, two-dimensional sections through soil in the form of a soil pit, a polished block, or a thin section.

Given a three-dimensional structural unit X , as illustrated in figure 3.17, we find some basic measures: the volume V , the total surface area S , the mean curvature C , and the total curvature K . The latter is a topological measure describing the connectivity.

In the more general context of integral geometry, these properties are defined for a d -dimensional structure through $d + 1$ Minkowski numbers M_k with $k \in [0, \dots, d]$. In the following, M_k are defined for $d = 3$. M_0 is the total volume of the structural unit

$$M_0(X) = V(X) \quad [L^3] \quad (3.2)$$

The other Minkowski numbers $M_{1, \dots, 3}$ are defined as integrals over the boundary ∂X of the structure X . Note that the boundary defines the object completely. Specifically we find the total surface as

$$M_1(X) = \int_{\partial X} ds = S(X) \quad [L^2] \quad (3.3)$$

where ds is the boundary element. The mean curvature is defined as

$$M_2(X) = \frac{1}{2} \int_{\partial X} \left[\frac{1}{r_1} + \frac{1}{r_2} \right] ds = C(X) \quad [L^1] \quad (3.4)$$

where r_1 and r_2 are the principle radii of curvature of the boundary element, which are positive for convex and negative for concave boundaries (fig. 3.17). Hence, the sign of the mean curvature changes correspondingly. Finally, the total curvature is given by

$$M_3(X) = \int_{\partial X} \frac{1}{r_1 r_2} ds = K(X) \quad [—] \quad (3.5)$$

The total curvature yields the value 4π for each closed convex boundary; the same is true for any closed concave boundary. In contrast, a closed saddle surface where r_1 and r_2 have different signs has a total curvature of -4π . Hence, the total curvature counts the number of isolated objects N (closed convex), the number of cavities H (closed concave), and the negative number of loops L (closed saddle surface). Consequently, $K(X)$ is directly related to the Euler number, which is defined as

$$\chi(X) = N - L + H = \frac{1}{4\pi} K(X) \quad (3.6)$$

Herewith, $\chi(X)$ is a positive number if, for example, X is composed by a number of isolated objects. If X is a connected network, it is a negative number that counts the number of meshes in the network.

The Minkowski numbers are basic measures in the sense of Hadwiger's theorem [13], which states that all other properties of a given structure that are motion invariant, continuous, and additive can be directly calculated from these numbers. In fact, most of the shape parameters used by Murphy et al. [21] can be derived from the Minkowski numbers. For a comprehensive discussion, see Mecke [19].

To compare images of different sizes it is convenient to relate the Minkowski numbers to the volume of the embedding space Ω^d to obtain Minkowski densities

$$m_{d,k}(X) = \frac{M_{d,k}}{\Omega^d} [L^{-k}] \quad k \in [0 \dots d] \quad (3.7)$$

In two dimensions, $d = 2$, we find only $d + 1 = 3$ Minkowski densities $m_{d,k}(X)$, which are defined in analogy to the three-dimensional case. Specifically $m_{2,0}$ is the area density, $m_{2,1}$ the density of the boundary length and $m_{2,2}$ the density of the two-dimensional Euler number. In 2D, there is only one principle radii of curvature and thus, no mean curvature is defined and the factor of proportionality between the total curvature and the Euler number reduces to 2π . Thereby, the two-dimensional Euler number is defined as $\chi_2(X) = N - L$.

A one-dimensional image of a structural unit consists of a transect that is part of the unit at some locations. Then, $m_{1,0}$ is the length density of the transect belonging to the structural unit and $m_{1,1}$ is the density of the one-dimensional Euler number counting the number of separated line segments. Finally, a zero-dimensional image is a point that belongs to the structural unit or not, so that the zero-dimensional Euler number $m_{0,0}$ is equal to 1 or 0, respectively.

A convenient property of the Minkowski numbers is based on the fact that they can be derived from samples of lower dimensions. This is of practical importance whenever a complete recording of a d -dimensional structure is not feasible and thus, we have to rely on samples of lower dimensions. In fact, for a given object, there is a well-defined relation between the Minkowski numbers obtained at subsamples of different dimensions. Such relations are the subject of stereology [30], which is based on integral geometry. Basically, we find the relation

$$m_{d,k}(X) = h_{d,k} m_{d-x,k}(X) \quad k \in [0 \dots d] \text{ and } x \in [1 \dots d] \quad (3.8)$$

where the coefficients $h_{d,k}$ are typically calculated for isotropic structures (or isotropic sampling strategies). The relations between the three-dimensional Minkowski densities and those of lower dimensions are summarized in table 3.1.

From this table some basic stereological formulas are evident: The volume density V_V corresponds to the area density A_A , the length density L_L and the point density P_P corresponds to a decreasing dimensionality of subsamples. For the surface density we find $S_V = 4/\pi B_A = 4I_L$, where $B_A = m_{2,1}$ is the length of the boundary related to the total area of a two-dimensional section and

TABLE 3.1
Relation Between the Three-Dimensional Minkowski Densities $m_{3,k}$ and Those of Lower Dimensions. The Coefficients, Which are Given Explicitly (for $m_{2,1}$, $m_{1,1}$ and $m_{2,1}$), are Only Valid for Isotropic Structures or Isotropic Sampling Strategies

$m_{3,k}$	Spatial dimension		
	2	1	0
$V_v(X) = m_{3,0}$	$m_{2,0}$	$m_{1,0}$	$m_{0,0}$
$S_v(X) = m_{3,1}$	$\frac{4}{\pi} m_{2,1}$	$4 m_{1,1}$	
$C_v(X) = m_{3,2}$	$m_{2,2}$		
$K_v(X) = m_{3,3}$			

TABLE 3.2
Two-Dimensional (top) and Derived Three-Dimensional Minkowski Densities (bottom) for the Structural Units of Figure 3.14

	Structural units			
	Root channels	Macropores	Cast infillings	Soil matrix
2D				
$m_{2,0}$ [–]	0.04	0.07	0.18	0.71
$m_{2,1}$ [cm ⁻¹]	4.12	2.53	4.17	11.2
$m_{2,2}$ [cm ⁻²]	30.6	1.97	3.95	–23.7
3D				
$m_{3,0}$ [–]	0.04	0.07	0.18	0.71
$m_{3,1}$ [cm ⁻¹]	5.25	3.23	5.31	14.3
$m_{3,2}$ [cm ⁻²]	30.6	1.97	3.95	–23.7
$m_{3,3}$ [cm ⁻³]		cannot be inferred from 2D		

$I_L = m_{1,1}$ represents the number of line segments inside the object related to the total length of the line. Finally, C_v equals the total curvature obtained from a two-dimensional section, $m_{2,2}$.

As indicated in table 3.1, some of the coefficients are only valid for isotropic structures. Specifically, this is the case if the measure depends on the spatial direction of inspection, which is true for the surface S and the curvature C , and if the subsample can have a preferred orientation, which is true for one- and two-dimensional subsamples. In natural systems, however, isotropy is rather the exception than the rule. To solve this problem, an isotropic sampling can be mimicked for digital image analysis, which ascertains the validity of the relations given in table 3.1. For more details, see Ohser et al.[23].

In table 3.2 the results for the Minkowski densities obtained for the various structural units in the thin section of figure 3.14 are shown. Additionally, the estimated three-dimensional properties are given.

Though the mean size of structural units, given by the ratio of volume and surface, or the mean connectivity can be obtained from the Minkowski densities, there is no information about the size distribution or the spatial distribution. This is because the Minkowski densities are integral measures of the entire boundary of a given structural unit. Hence, a more comprehensive description is desirable. This can be obtained through an extension using the information on distances between single components of a structural unit, as demonstrated in the following section.

3.4.3 EXTENSION TO MINKOWSKI FUNCTIONS

A more detailed quantitative description of the morphology for a given structural unit is obtained by analyzing the distribution of distances within a structural unit or the distances within the background. If the boundary line or surface is again chosen as a reference, each spatial coordinate has a well-defined distance to this boundary. To measure the corresponding distance distribution we use some fundamental tools of mathematical morphology [26] (i.e., erosion and dilation).

A morphological erosion is defined with respect to a so-called *structuring element* B of arbitrary but well-defined size and shape. To evaluate distances we choose isotropic structuring elements (i.e., circles and spheres in 2D and 3D, respectively). Then, considering the structural unit X , its erosion is given by

$$X_e = \{x : B_x \subset X\} \quad (3.9)$$

In other words, this means that the eroded structure X_e is given by all locations x where the structuring element B_x (B located at x) is completely part of X . This is illustrated in figure 3.18 where all points within the hatched area belong to the erosion with respect to a circular structuring element with a given radius r . Evidently, the hatched area describes all locations inside the structural unit where the distance to the boundary is larger than r .

Morphological dilation operates outside of the structural unit and is defined as

$$X_d = \{x : B_x \cup X \neq \emptyset\} \quad (3.10)$$

Herewith, the dilation is given by all locations where the structuring element with radius r intersects the structural unit. In figure 3.18 this is true for the gray-shaded area in addition to the structural unit itself. Hence, dilation of X corresponds to an erosion of its complement. Evidently, the complement of the dilated structure describes all locations outside the structural unit where the distance to the boundary is larger than r .

By using structuring elements of various sizes, it is possible for each location or pixel in a digitized image to assign the distance to the boundary. Hence, the binary image of a structural unit

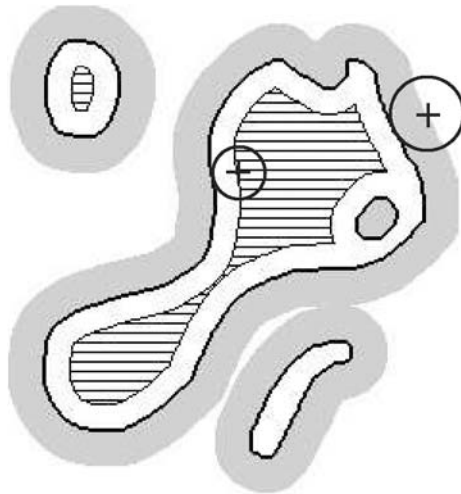


FIGURE 3.18 Erosion and dilation of a structural unit (black outline) using structuring elements. The eroded (hatched) and the dilated structure (original structure + gray area) depends on the size of the structuring element (circles).

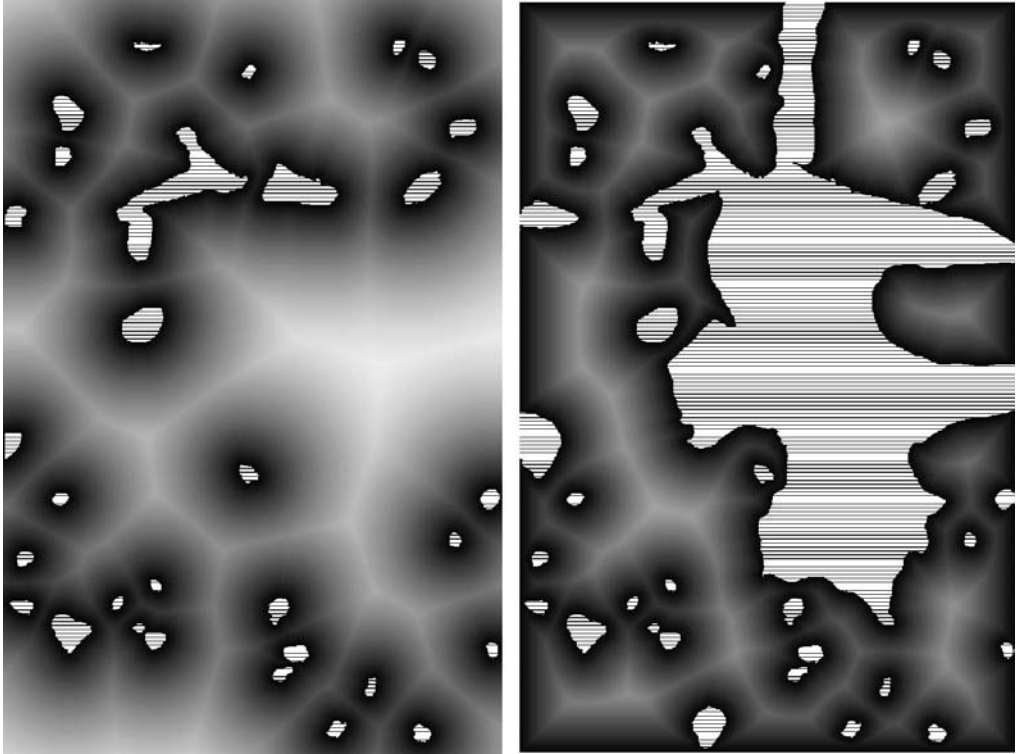


FIGURE 3.19 Distance maps derived from figure 3.14 for dilations of the root channels (left) and for erosions of the porous matrix (right). The gray level of each pixel corresponds to its distance to the boundary of the structural unit.

can be transformed to a grayscale image where the gray level corresponds to the distance to the boundary. In figure 3.19 such *distance maps* are shown for the distances between root channels and the distances within the porous matrix of figure 3.14.

Based on such distance maps, the Minkowski numbers can be evaluated as a function of distance. The binarization of a distance map according to a gray threshold corresponds to a segmentation according to a certain distance. Minkowski numbers obtained for a complete series of distances are continuous functions, which we will refer to as Minkowski functions according to Mecke [19]. They exhibit a characteristic shape and include valuable information on the size and the spatial distribution of a structural unit.

As an example we calculated the Minkowski functions for the distribution of savanna within the landscape of semiarid Chaco shown in figure 3.13. Thereby, we compare the results for the years 1975 and 2001. The analysis is restricted to the central catchment of about 800 km². As shown in figure 3.20, the total area of savanna decreased considerably during this period, which is a consequence of the intensified agricultural activities.

The Minkowski functions provide an extensive quantification of the observed distribution patterns. Figure 3.21 shows the densities of the area, boundary length, and Euler number for the different years. The values for zero erosions/dilations correspond to the Minkowski numbers of the original image (fig. 3.20). Eroded subsets of this image are indicated with a negative sign while dilations are positive. The values correspond to the radius of the circular structuring element, which was used to calculate the distance map. The unit is “number of pixels,” the size of which is about 30 m in this case.



FIGURE 3.20 Distribution of savanna (black) within a catchment in the semiarid Chaco in Argentina as obtained from Landsat data in 1975 (left) and 2001 (right).

The reduced area density of savanna in 2001 is obvious. Moreover, the size of individual patches is small. This is quantified by the area of savanna, which vanishes almost completely after three erosions, corresponding to a maximum diameter of individual patches of about 210 m. The linear increase of the area density with dilations in 2001 is due to the sparse distribution of the individual patches. In 1975 there is still a considerable density of savanna after 10 erosions and the density reaches almost 1 after 20 dilations. This means, there is a considerable amount of closed, continuous fields of savanna larger than about 600 m (20×30 m/pixel) and the largest patches of other types of vegetation is about 1200 m. Thus, the Minkowski function of the area density provides valuable information on the size distribution of continuous entities of a structural unit.

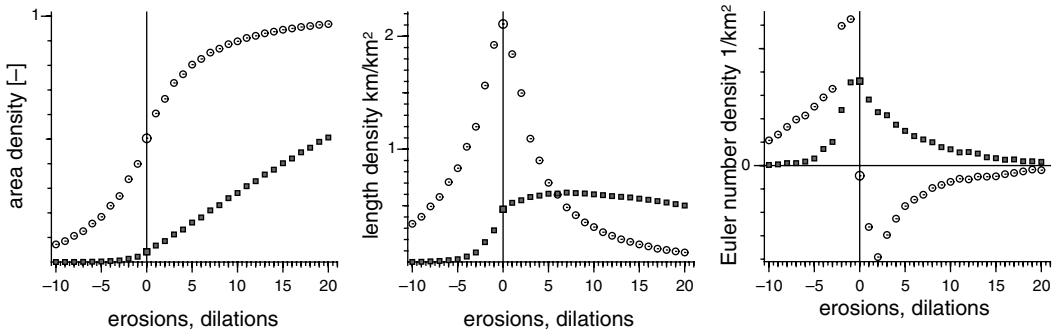


FIGURE 3.21 Minkowski functions of savanna as shown in figure 3.20 for the year 1975 (open circles) and 2001 (gray squares).

The Minkowski function for the boundary length contains similar information. It reflects the slope of the area density function since the change of area with erosion or dilation is directly related to the boundary length.

The density of the Euler number is positive for 2001 because there are only isolated patches of savanna. The decreasing Euler number with dilations provides information about the distance distribution between individual patches. In 1975, the Euler number is negative, which indicates a well-connected structure. Remarkably, the Euler number gets positive after the first step of erosion, which indicates that the spatial connectivity is given by narrow paths of some 60 meters (corresponding to 2 pixels in the image). On the other hand, after the first two steps of dilation the Euler number decreases considerably, which means that the spatial extent of other vegetation types is small.

In summary, the most important morphological aspects with respect to the quality of structural units as natural habitats are quantitatively captured by the Minkowski functions. Thus, Minkowski functions may serve as a quantitative criteria to evaluate the quality of structural units or to compare the results of structure models with direct observations.

3.5 CONCLUSIONS AND FINAL REMARKS

In this contribution we tried to highlight the complex structure of natural systems at various spatial scales with special emphasis on soil. The observable diversity is the prerequisite and, to a significant extent, also the corollary of the observable biodiversity. This was qualitatively demonstrated through some examples in this chapter and is obvious from manifold publications in the field of soil micromorphology.

The spatial structure of natural systems plays a central role for most environmental processes and its complexity is the main hurdle for a quantitative understanding of natural systems. This constitutes the impetus to also get quantitative access to the subject. The approach of Minkowski numbers and Minkowski functions was already applied successfully to derive physical properties of soil from the structure of the pore space [29]. An extension of this approach toward other environmental aspects suggests itself.

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4 Microbial Communities Introduced through Organic Amendments and by Air-Transport into Agricultural Soils

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4.1 INTRODUCTION

Organic wastes like animal manures, sewage sludges, composts, or fermented harvest residues are increasingly produced and recycled in agricultural landscapes all over the world. In a countermove, the areas for food production significantly decreased during the last decades due to increasing agricultural productivity. As a result, the agricultural areas taking up the increasingly produced wastes for recycling are decreasing. The microorganisms introduced to the agricultural soils by wastes may cause shifts in the composition of the autochthonous soil microflora and fauna and eventually change soil functioning.

As an example, one of the about 13.6 million German cattle produces daily approximately 35 kg of manure [1], or 12.7 tons per year. The cattle-produced amount of manure is almost doubled by the 2.6×10^7 German pigs and 1.1×10^8 chickens [2] and annually the residues of the German waste water treatment and municipal composting plants add about 7.8×10^6 plus about 3.8×10^6 tons (dry weight), respectively, to the agricultural systems [3].

Supposing that about 3×10^8 tons of the annually produced organic wastes are homogeneously distributed on the about 17 Mio ha arable land in Germany, then each m^2 of agricultural soil would be enriched by the microbial biomass of about 1.76 kg organic material, which corresponds to the recommendation of compost fertilization by the “Deutsche Bundesgütegemeinschaft Kompost” (<http://www.rgk-suedwest.de>). Under the further assumption that one gram of organic waste material contains 10^{10} microbial cells, then the upper 0.15 m^3 arable land (bulk soil density of 1.5 t per m^3) would be enriched by organic manure amendment with about 10^{13} pathogenic and nonpathogenic microbial cells. This would mean that about 0.78% of the microflora already present in the 0.15 m^3 arable land are added by organic amendment.

The microbial activities in relation to catabolism in soils are quite diverse. Thousands of chemical and biochemical compounds are involved in that catabolism, which can be divided in (1) primary compounds that are directly derived from other organisms, plants, microorganisms, or animals, and (2) secondary compounds, which are produced during the complex organic mineral soil interactions (often enzymatically catalyzed) and resulting in small or (sometimes) extensive changes in chemical bonds and chemical structures.

The most important “players” in the turnover process of organic compound in soils are microbial populations (bacteria and fungi). They are biochemically and also phylogenetically extremely diverse and are undoubtedly the most numerous organisms on earth and have been estimated to make up 2.5×10^{29} cells (numbers in soils of all biomes). These estimates translate into 2×10^9 to 10^{10} cells per gram in the top meter and 10^8 cells per gram in the 1- to 8-m soil depth [4].

In soils away from the rhizosphere, the environment for bacteria is usually stressful. In the last years it has been found that a majority of bacteria exist in this low-nutrient condition under starving conditions [5]. Although some bacteria can double every 20 minutes or less in growth media in the laboratory, in nutrient-limited environments they may undergo only two to three divisions per year, on average, in soil under field conditions because of the extreme limitations of available reduced carbon substrates.

Knowledge of the extensive diversity of prokaryotes has increased greatly in the past two decades. The principal concern of microbial ecology is to trace both the extent of species of bacteria, as well as the archaea. Until recently, archaea were considered to be inhabitants of extreme environments, but they have been also found in numerous other habitats, including fresh water lakes and forests, agricultural soils, and composts [6–8]. For more information on soil prokaryote interactions in soils and rhizospheres, the review by Kent and Triplett [9] is highly recommended.

After introduction of molecular methods into microbial ecology, one method often used to analyze bacterial populations is to amplify DNA extracted from environmental samples by polymerase chain reaction (PCR), using primers universal to the 16S ribosomal RNA (rRNA) genes of bacteria and archaea [10,11]. In principal, either DNA or RNA can be extracted from soils, but a majority of the studies have been focused on DNA extraction, which is easier to accomplish because of the higher lability and turnover of RNA.

Sophisticated techniques are now available to analyze microbial community structure and function, by analyzing microbial rRNA and mRNA, respectively. Both types of RNA can be extracted from soils and converted to complementary DNA (cDNA) by the enzyme reverse transcriptase for subsequent PCR amplification.

Despite the increased application of new molecular methods for studying the microbial ecology of soils, the distribution and abundance of microorganisms in soils is often so patchy that it is almost impossible to make an accurate determination of their mean abundances without dealing with a very high variance about that mean, when viewed on a macro scale. This has been reviewed by Coleman et al. [12]. This is also the major reason why, up til now, very little is known about the microbial community structure in these untreated or treated organic wastes. More details of the methodological drawbacks are given below. Accordingly, this chapter tries to review the most abundant microbial communities present in waste materials that are considered for agriculturally recycling.

4.2 MICROORGANISMS IN ORGANIC WASTES

The organic residues introduced to arable lands comprise an extensive variety of monomeric and polymeric chemical structures. The 35 or more bacterial phyla residing in soils have evolutionarily developed many strategies to mineralize all the mono- and polymer-organic compounds reaching the soils (see chapter 15 in this volume). The soil bacteria are working synergetically together with other microfloral and faunal members for recycling the introduced organic compounds, even the recalcitrant ones that need larger time windows for being completely degraded (see chapters 4–9,

15, and 18 in this volume). Thus, the microfloral and -faunal consortia residing in different soil niches (see chapters 2, 4–12 in this volume) are able to recycle the introduced waste materials.

4.3 MICROBIAL COMMUNITY IN COMPOSTS

During composting animal manures or organic kitchen and garden wastes are microbiologically transformed into CO₂, H₂O, and residues that are not degraded during the 10 to 12 weeks of processing. Due to the separate collection of organic wastes in Germany the composting and biogas production plants inreased in parallel to the separate collected organic waste.

More than 600 German full-scale composting plants presently produce nearly 6–7 million tons of compost annually [13].

Although there is an extensive increase in this waste processing, very little is known about microbial communitiy structure at the different stages of composting for reasons given above. Culture-based approaches have previously been used to study acitve microorganisms during the different composting stages. A selection of those results is shown in table 4.1. However, a small fraction of microbes present in environmental samples are typically culturable [14]. In contrast to those time- and material-consuming cultivation-based methods, cultivation independent fingerprint methods allow the analysis of higher sample numbers. Methods like PLFA, DGGE, SSCP, or T-RFLP therefore have the potential to show community changes in the compost material [15–18] also including members of uncultured microorganisms.

In a study on community changes in waste material introduced into the composting process, Michel et al. [18], for example, showed with T-RFLP analysis that primarily gramnegative α , β , γ Proteobacteria are the abundant representatives of the community. However, after the first few days they detected a rapid decrease of these organisms and the community composition was then composed mainly of members of the grampositives, the Bacillus–Clostridium branch and the Actinobacteria. The latter bacterial groups are expected to be the dominating bacteria during the so- called thermophilic phase (table 4.2). The thermophilic phase starts due to a rapid degradation of the huge amounts of easily available and energy-rich organic compounds. Often temperatures as high as 60–80°C are reached. Thermophilic and thermotolerant microorganisms are now dominating and start to degrade the polymer structures of the compost material like celluloses, hemicelluloses, and proteins. Besides

TABLE 4.1
Microbial Species Changes and Their Percent Proportion During Composting

Phase of Composting	Mircrobial genera (species)	Percentage of the total microbial population density	References
Mesophilic	<i>Bacillus</i> , <i>Proteus</i> , <i>Micrococcus u.a.</i>	10–20	[56]
	<i>Bacillus</i> , <i>Alcaligenes</i> , <i>Pseudomonas</i> , <i>Aeromonas</i>		[57]
	<i>Penicillium</i> , <i>Aspergillus</i> , <i>Mucor</i>		[57]
Thermophile	<i>Geobacillus</i> , <i>Thermobacillus</i>	71–92	[57]
	<i>Thermoactinomyces</i> , <i>Thermomonospora</i> , <i>Saccaropolyspora</i> ,		[58]
	<i>Saccharomonospora</i>		
	different <i>Thermus</i> strains		[59]
	<i>Methanobacterium thermoautotrophicum</i>		[34]
Cooling and maturation	<i>Scytalidium thermophilum</i>		
	<i>Humicola insolens und Humicola grisea var. thermoidea</i>		[60]
	<i>Aspergillus fumigatus</i>		
	<i>Ascomycetes</i> , <i>Basidomycetes</i>		
	<i>Methylocaldum szegediense sp.</i>	~ 1	[36]

TABLE 4.2
Apparent Diversity in Cattle Rumen, Manure, Separator, and Lagoon Water

Cattle	Phylum		Closest relatives (number of clone)	Similarity (%) based on 16S rDNA
Rumen Solid/Fluid	LGC (49)	58.3%	<i>Butyrivibrio crossotus</i> (3)	90–91%
			<i>Butyrivibrio fibrisolvens</i> (8)	93–99%
			<i>Selenomonas ruminantium</i> (1)	94%
			<i>Eubacterium halii</i> (2)	93%
			<i>Eubacterium ventriosum</i> (1)	90%
			<i>Termitobacter aceticus</i> (1)	90%
			<i>Clostridium celerecrescens</i> (2)	90%
			<i>Clostridium xylanolyticum</i> (1)	90%
			<i>Ruminococcus flavefaciens</i> (2)	95–96%
			NA (28)	
	Bacteroidetes (28)	33.3%	<i>Prevotella ruminicola</i> (14)	91–95%
			NA (14)	
	Proteobacteria (2)	2.4%	NA (2)	
	Mycoplasmas (2)	2.4%	NA (2)	
	Spirochaetes (2)	2.4%	<i>Treponema bryantii</i> (2)	97–98%
Manure [1]	NA(1)	1.2%	NA (1)	
	LGC	77.0%	<i>Clostridium lituseburense</i> (31)	97–99%
			<i>Ruminococcus bromii</i> (15)	96–99%
			<i>Bacillus silvestris</i> (12)	97%
			<i>Eubacterium tenue</i> (13)	99%
	Actinobacteria	9.0%	n.i.	
	Bacteroidetes	7.0%	n.i.	
	Proteobacteria	5.0%	<i>Acinetobacter johnsonii</i>	
	Cyanobacteria	0.7%	n.i.	
	Spirochaetes	0.4%	n.i.	
Separator [1]	LGC	80.0%	<i>Clostridium lituseburense</i> (69)	96–99%
			<i>Clostridium celatum</i> (8)	96–98%
			<i>Eubacterium tenue</i> (31)	97–100%
			<i>Turicibacter sanguinis</i> (18)	99–100%
			<i>Salinicoccus roseus</i> (14)	96–99%
			<i>Alkalibacterium olivoapovlitiicus</i> (23)	92–96%
			<i>Lactosphaera pasteurii</i> (8)	95–98%
			<i>Bacillus thermocloacae</i> (12)	91–92%
	Actinobacteria	10.0%	n.i.	
	Proteobacteria	5.0%	n.i.	
	Bacteroidetes	4.0%	n.i.	
	Chloroflexi	0.03%	n.i.	
Lagoon water [1]	LGC	77.0%	<i>Clostridium lituseburense</i> (99)	97–99%
			<i>Clostridium aminobutyricum</i> (13)	93–94%
			<i>Eubacterium tenue</i> (47)	98–100%
			<i>Turicibacter sanguinis</i> (13)	99–100%
			<i>Streptococcus suis</i> (11)	92–94%
			<i>Thiocapsa rosea</i> (11)	95–96%
	Proteobacteria	15.0%	<i>Achromatium</i> sp. (10)	93–94%
	Actionbacteria	3.0%		
	Bacteroidetes	3.0%		
	Planctomycetes	1.0%		
	Chloroflexi	0.003%		

these bacterial groups like *Bacillus/Clostridium* and *Actinomyces mucoraceous* fungi (e.g., *penicillium* spp. and *aspergillum* spp.) are increasingly found during the thermophilic phase [19–24].

Besides the clear temperature increase generally the compost acidifies to a pH of 5 to 6 within the first few days, which can be expected due to an acidification caused by microbial fatty acid formation [25]. The clear pH-increase following the acidification can be attributed to degradation of fatty acid in combination with an intensive mineralization of nitrogen, coupled with the production of ammonia [25].

Those rapid substrate changes and shifts in environmental conditions like temperatures seems to favor those observed shifts in microbial communities as reported by Michel et al. [18].

Due to the fact of a restricted substrate availability, the compost heaps start to cool down because heat dissipation surpasses heat production. Mesophilic microbial consortia consisting of various fungi as *Aspergillus candidus*, *Dactylaria* sp., *Mucor* sp., *Scopulariopsis* sp., *Trichothecium* sp., *Verticillium* sp., *Acremonium* sp., *Cephliophora* sp., *Geotrichum candidum*, and *Scopulariopsis breviacaulis* [26] as well as an increasing detectable bacterial diversity (members of up to seven phylogenetic groups) was shown by T-RFLP analysis in stabilized compost [18]. This phenomenon is expected as the so called “recolonization” by thermotolerant fungi and bacteria occur as well [23].

DNA-fingerprinting techniques (e.g., DGGE profiles) are increasingly applied for illustrating the important role of changing environmental conditions on the development of microbial communities in composts and finally in the compost-amended soils.

However, the following brief methodological excursion reveals that more than one method should be used to study the microbial composition in compost heaps. Having extracted DNA from mature compost developed from cow manure, Green et al. [27] found by DGGE profiles and cloning (for methodological details, see chapters 6 and 16 in this volume) that the dominating bacterial phylum in cow manure compost is *Bacterioidetes*. Not detectable by this technique was the expected phylum *Actinomyces*. Narihiro et al. [28] included DGGE and quinone profiling, another method for detecting bacteria in complex substrates, into their compost studies and found both *Bacterioidetes* and members of the phylum *Actinobacteria*; the latter, however, only by quinone profiling.

Although composting is expected as a degradation process under oxic conditions, methane emissions from compost piles range between 0–119 g CH₄ m⁻² d⁻¹ [25,29–33]. These emissions indicate that a considerable number of *Archaea* responsible for methane formation must be present.

The raising temperatures that lead to a decrease of oxygen solubility is prerequisite for thermophilic methanogenic activity in oxygen-depleted zones, which even in aerated heaps can develop. It is therefore not surprising that in compost piles prepared from wastes of mushroom culturing, 2×10^8 thermophilic methanogens consisting mainly of the species *Methanobacterium thermoautotrophicum* (synonym: *Methanothermobacter thermoautotrophicum*) [34] were detected.

The study by Cahyani et al. [35] is one of the rare reports describing the succession and composition of *Archaea* during the composting process of rice straw. The authors presume that methanogenic *Archaea* are mortified by high temperatures during the thermophilic phase of rice straw composting. However, not yet published cloning experiments for *Archaeal* communities after DNA extraction from compost material of methane-emitting piles reveal rapid changes between thermophilic *Methanosarcina*, *Methanothermobacter*, and members of up to now unculturable *Archaea* species (unpublished own results). Recently a moderate thermophilic methanotroph with a high methane oxidation rate could also be isolated from a municipal composting plant. The methylotrophic bacterium with an in situ concentration of about 10^6 cells per gram seems to be closely related to the thermophilic species *Methylocaldum szegediense* [36].

In conclusion, pile dimension [25], pile density [37], and the consistence of input material [38] as well as the composition of methanogenic and methanotrophic community [41] seem to be the obvious factors regulating the emission from compost piles. These latter findings, together with the fast changing gradients inside the compost heaps, suggest that the compost microflora is certainly more diverse as expected and existing results presumably give only a small picture about the real microflora in this habitat.

4.4 AIRBORNE MICROORGANISMS RELEASED BY COMPOSTS

Though air is not a habitat for microorganisms to live in (low humidity, lack of nutrients), numerous microorganisms can be isolated from air samples. The microorganisms found in air samples are originally released from composts, waste waters, animal intestinospheres, surface waters, phyllospheres or soils, or other sources. Attached to solid particles or small waterdrops, microorganisms are transported with the air flow. Due to various evolutionarily developed resistance mechanisms, microorganisms can persist in the air for several minutes [37,38] or over much longer periods, as particle transports from the Sahara to North America reveal.

Processes like active aeration and turning around of compost heaps can lead to large amounts of microorganisms emitted into the air and transported into the adjacent biotops (e.g., agricultural soils). Depending on wind direction and speed bioaerosols, transport distances of more than 1 km can be achieved [39]. A mean distance transport of 2.5 km from each of the 48 presently running composting plants in the German State Hesse (Bundesgütegemeinschaft Kompost) assumed reveal that compost microorganisms may reach an area of 940 km². Compared to the 21,000 km² land area of the State Hesse compost, microorganisms reach regularly about 4.5% of the agricultural sites.

It has been known for a long time that bioaerosols originating from compost or composting facilities can contain microorganisms such as vegetative cells and spores of bacteria and especially of actinomycetes and fungi [19]. The cells, spores or hyphae are often attached to each other as aggregates or bound to organic particles [40]. The vast majority of the airborne microorganisms (with the exception of some Gram-positive bacteria and fungal spores) are rapidly inactivated as a result of desiccation, temperature increase, or UV radiation [41]. Because of these environmental stress factors, viable and culturable fractions represent only a small part of the total microorganisms present in bioaerosols.

Besides the nonpathogenic and pathogenic microorganisms of different groups and taxa emitted from solid and liquid animal excrements, compost or waste water treatment plants are also microbial metabolites with distinct odors released from the above facilities and transported by air [21,22,24,42,43]. Obviously, there is a close relationship between the released spectrum of airborne microorganisms and the emitted odor compounds, revealing that the air-transported community represents closely the actual stage of the developing emittent. The spectrum of microfungi emitted from compost heaps at different developing stages is one of the proofs of the above relationship [22].

SSCP fingerprints from heap material taken right before the turning around of a heap and the microorganisms in air samples collected after the turning around of a heap resulted in a high similarity between both data sets (Jäckel et al., in preparation).

4.5 ANAEROBIC DIGESTERS

Instead of composting, organic wastes originating from agriculture, forestry, the food industry, or municipal and industrial waste water treatment plants can be decomposed by a complex microbial community of hydrolytic, fermenting, homoacetogenic, syntrophic, and methanogenic microorganisms before they are agriculturally recycled. End-products are acids, CO₂, and finally methane [44–47; see chapter 16 in this volume].

Anaerobic waste digestion is frequently combined with biogas production and the residues, not converted into biogas, are agriculturally recycled. The first degradation step in the anaerobic kept biogas reactor is the exoenzymatic hydrolysis of cellulose and the other polymeric biomolecules into the monomer subunits, which are then transformed by fermentating bacteria under energy gain into fatty acids (e.g., propionate, acetate, butyrate), alcohols, CO₂, and H₂. Syntrophic bacteria decompose, the alcohols and fatty acids turn to acetate, and the methanogenic consortium of Archaea form finally out of acetate, CO₂, and H₂ methane [48; see chapter 16 in this volume].

Fermenting bacteria and the methanogenic archaea of about 10⁵–10¹⁰ cells per ml sludge are the dominating microorganisms in anaerobic digesters or biogas-producing plants. Besides a variety

of fermenting bacteria are typical methanogenic isolates: *Methanobacterium thermoautorophilum*, *M. wolfei*, *M. formicum*, *M. bryantii*, *M. thermoalcaliphilum*, *Methanobrevibacter arboriphilus*, *M. ruminantium*, *M. smithii*, *Methanomicrobium* sp., *Methanospirillum hungatei*, *Methanogenium aggregans*, *M. bourgense*, *Methanosarcina barkeri*, *M. thermophila*, *M. mazei*, *Methanotherix soehngenii*, *M. concilii*, *M. thermoacetophila*, *Methanoplasma elizabethii*, and *Methanosaeta* spp. [49,50]. Only very few detailed and comprehensive studies on microbial communities and their changes have been published until now [51].

Molecular microbial diversity studies in an anaerobic digester, employing 16S rDNA sequencing and the analysis of the 146 operational taxonomic units (OTUs), revealed that, besides various methanogens mentioned above, microorganisms assigned to the domain bacteria with a close relationship to the low GC-Grampositive phylum (34% of the OTUs) were present [52, table 4.1]. A relatively high percentage (26% of the OTUs) belongs to the phylum *Bacteroidetes*. The *Proteobacteria* comprised 17% of the analyzed OTUs. The bacterial 16S rDNA sequence similarities of the organisms to established taxa was in most cases below 97% [53]. High 16S rRNA similarities of the isolates were found with the members of the genus *Bacteroides* (e.g., *Bacteroides eggerthii* and *Bacteroides zoogloformans*), and of the low GC Grampositive phylum *Enterococcus saccharolyticus*, *Enterococcus faecalis*, *Clostridium propionicum*, *Clostridium barkeri*, and *Clostridium ramosum*. The isolates of the phylum *Proteobacteria* were most closely related to *Desulfovibrio* sp. and *Enterobacter* (*Klebsiella*) *aerogenes*.

4.6 DAIRY WASTES AND MICROBIAL DIVERISTY

Ruminants are often kept on cement floors and their droppings are rinsed by water into separator pits where liquid and solid particles are separated [1]. The solids are either directly brought to the fields or stored for certain periods on dung heaps. The liquid urine-water proportion is often stored in a pit, before spreading on arable land. Occasionally, the outflow of dairy farms is treated in constructed wetlands for reducing the coliform bacteria, nitrates, and the biological oxygen demand [54].

Fresh solid and liquid animal wastes, if directly applied to an agricultural site, confront the autochthonous soil microflora with bacteria, fungi, and protozoa enriched in the gastrointestinal tract (see chapter 18 in this volume). Excrements may contain pathogenic bacteria like *Campylobacter* spp., *Salmonella* spp., or *Mycobacteria* spp. in higher numbers. In these cases they are of public concern because of possible drinking water contaminations and impaired livestock production. Diversity analysis based on 16 S rDNA sequencing showed that representatives of low GC Grampositives and *Bacteroidetes* are dominating organisms in solid and fluid animal wastes (table 4.2) Both bacterial groups are characterized by a high diversity of species. Of concern are also the methane-producing archaea (methanogens) present in high numbers in the rumen. They serve as H₂-absorbers and belong in most cases to the genera *Methanobrevibacter*, *Methanobacterium*, *Methanomicrobium*, and *Methanosarcina* [55] (see chapter 18 in this volume).

Surprisingly, the molecular isolates from stored animal fluids and the gastrointestinal tract reveals 16S rDNA-sequence similarities below 97%, whereas those obtained from the animal manures show similarities mostly above 97%, which are more reliable to establish taxa (table 4.2). The low GC Grampositives are the predominant phylum in solid manures, whereas *Bacteroidetes* strains occur only in low numbers. The closest relatives to the low GC Grampositives of the solid wastes are *Clostridium lituseburense*, *Clostridium aminobutyricum*, *Clostridium celatum*, *Eubacterium tenue*, *Ruminococcus bromii*, *Turicibacter sanguinis*, *Salinicoccus roseus*, *Alkalibacterium olivoapovlitis*, *Lactosphaera pasteurii*, *Bacillus thermocloacae*, *Bacillus silvestris*, and *Streptococcus suis*. Treated pig manures seem in contrast to the wastes of the ruminants to comprise a completely different microbial community (table 4.3).

In conclusion, with the different animal waste materials, completely different microbial consortia are brought to the agricultural fields. Dung piles receiving fresh material daily comprise differently aged straw-animal-excrement mixtures. Relatively fresh animal manure may in its

microbial diversity be closer to the microbial consortium of the gastrointestinal tract of cattle, while a straw–animal–excrement mixture of a month or older is, rather, approaching a microbial complexity similar to that of maturing compost heaps (table 4.2, table 4.3 and table 4.4).

TABLE 4.3
Apparent Diversity in Pig Gastrointestinal Tract, Different Manure Treatments

		Pylogenetic groups/ closest relatives (number of clones)	Number of detected phylotypes	16S rDNA-based similarity (%)	Reference
Gastrointestinal tract of pigs	LGC (74%)	<i>Eubacterium</i> and relatives	125	93.0*	[61]
		<i>Clostridium</i> and relatives	109	92.2*	
		<i>Bacillus-Lactobacillus-Streptococcus</i> subdivision	46	96.7*	
	<i>Bacteroidetes</i> (12.2%)		42	87.5*	
	<i>Proteobacteria</i> (12%)		20	94.8*	
	<i>Sporomusa</i> and relatives		15	94.7*	
	<i>Mycoplasma</i> and relatives		8	78.6*	
	High G+C grampositives		4	93.5*	
	<i>Spirochaetes</i> and relatives		2	86.4*	
	<i>Clostridium purinolyticum</i>		1	94.4*	
	<i>Planctomyces</i> and relatives		1	86.0*	
	<i>Flexistipes sinuaravici</i>		1	85.9*	
	<i>Anaerobaculum thermoterrenum</i>		1	84.3*	
Pig manure stored unaerated (mesophilic)	<i>Clostridium butyricum</i>			99	[62]
	<i>Clostridium disporicum</i>			98	
	<i>Spirochaeta</i> sp.			90	
	<i>Pedobacter</i> sp.			91	
	<i>Rhodanobacter</i> spp.			92–95	
	3× Unidentified			89–93	
	<i>Eubacterium</i>				
	Uncultured delta <i>proteobacterium</i>			86	
	Uncultured rumen <i>bacterium</i>			88	
	3× Uncultured bacterium			86–94	
Pig manure stored aerated (mesophilic)	<i>Bacillus thuringensis</i>			94	
	<i>Sphingobacterium</i> sp			90	
	<i>Sphingobacterium</i> -like organism			93	
	<i>Paenibacillus</i> sp.			95	
Aerobic thermophilic batch reactor	<i>Bacillus thermocloacae</i>				[63]
	<i>Schineria larvae</i>				
	<i>Bacillus</i> spp				
	<i>Lactobacillus crispatus</i>				
	<i>Clostridium butyricum</i>				
	Unidentified <i>Clostridia</i>				
	Unidentified <i>Bacteroides</i>				
		Unidentified <i>Spirochaetes</i>			
		<i>Pseudomonas</i> sp.			

TABLE 4.4
Apparent Diversity in an Anaerobic Digester

Domain distribution [52]	Phyla or subclass (%)	Genera or species
Bacteria (133)	LGC (34)	
	<i>Bacterioidetes</i> (26)	<i>Bacteroides</i>
	Alpha-, Beta-, Gamma-, Delta-, Epsilon-, Proteobacteria (17)	
	HGC (6)	
	<i>Spirochaetes</i> (4)	
	<i>Planctomyces/Chlamydothrix</i>	
	<i>Synergistes</i>	
	N.A.	
		<i>Methanosarcina barkeri</i>
		<i>Methanosarcina frisia</i>
Archaea (6)		<i>Methanobacterium formicicum</i>
	Unknown <i>Crenarchaeota</i>	
Eucarya (7)	Unknown <i>Thermoplasma</i>	
	<i>Hemiascomycetes</i> (29.8)	
	<i>Parabasallidae</i> (37.2)	
	<i>Hemiascomycetes</i> (16)	
	<i>Acantamoebidae</i> (9.6)	
	<i>Phreatamoebids</i> (4.3)	
	<i>Phreatamoebids</i> (1.1)	
	<i>Porifera</i> (2.1)	

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5 Discerning the Diversity of Soil Prokaryotes (Bacteria and Archaea) and Their Impact on Agriculture

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5.1 PROKARYOTES IN SOIL: A STORY OF SUCCESS

Prokaryotes are the most successful organisms on our planet, as they outnumber every other group of organisms. The total amount of carbon and nitrogen stored in the prokaryotes is in the same range of the amount that is located in plant biomass above Earth;¹⁴⁰ and prokaryotes are active: for soil, it has been estimated that the average turnover of elements within this prokaryotic biomass is 2.5 years.¹⁴⁰ The driving force for this turnover on the global scale is the input of energy that initially comes from sunlight and photosynthesis, and that is ultimately lost by heat radiation. The primary and major source of carbon and energy for all soil-inhabiting organisms comes from the plants. The energy is first captured chemically by the synthesis of high energy compounds, mainly ATP (adenosine triphosphate) and this synthesis is driven by redox-reactions in which electrons are transferred from an electron donor to an electron acceptor. The flow of energy in soil supports the development of the highest diversity of life that we find on Earth, and most of this soil biodiversity is represented by prokaryotes.

Prokaryotes are microorganisms that are characterized by a common cell structure. In contrast to more highly evolved eukaryotes [*Eukarya*], they are normally smaller, and they lack a cell nucleus surrounded by a membrane. There are two major prokaryotic groups also known as domains or superkingdoms (i.e., *Bacteria* and *Archaea*). It is still under debate how the three groups, *Bacteria*,

Archaea, and *Eukarya*, evolved.⁸³ Consequently, there is not yet a definite answer to the question of whether *Prokarya* and *Eukarya* are the two separate domains of life or whether each group, *Bacteria*, *Archaea*, and *Eukarya*, should be regarded as a domain.^{70,114,143}

To introduce the biodiversity of soil microorganisms, it makes sense to separate “soil prokaryotes” from small soil eukaryotes, since bacteria and archaea have similar cell sizes, a mostly unicellular lifestyle, and, in addition, both are metabolically more versatile than the eukaryotic microorganisms. It is this versatility, combined with the high, metabolically active surface area compared to their cell volume, that make soil prokaryotes so important for the geochemical cycles in terrestrial ecosystems. In fact, there are several steps in the carbon and nitrogen cycles in soils that can only be performed by prokaryotes (e.g., the biogenic synthesis of methane or the binding of molecular nitrogen from the atmosphere [nitrogen fixation]). In addition, many different soil prokaryotes are able to degrade plant residues, pesticides, and other xenobiotic compounds and thereby lay the foundation for soil fertility and sustainability of cropping systems.

Due to their small size and their metabolic versatility, soil prokaryotes are perfectly equipped to colonize any imaginable ecological niche of a terrestrial ecosystem. Soils of all climatic regions are inhabited by prokaryotes, as these organisms have adapted to different concentrations of nutrients, a wide range of substrates, and ambient temperatures. Soil prokaryotes have also adapted to water-stress and high salinities and, depending on the redox potential, different organic and inorganic compounds can be used as electron donors and acceptors. There is sufficient evidence that even deep subsurface soils with almost no inflow of nutrients have a considerable prokaryotic biomass. The average turnover time under these conditions, however, may be as low as 1,000 to 2,000 years.¹⁴⁰

The physical properties of soil allow for the coexistence of many different microbial habitats within only short distances (e.g., within a soil aggregate or on particles of organic matter). Prokaryotes also colonize other organisms and interact with them. Specific relations exist with plants, especially in the rhizosphere (see chapter 10 in this volume). Soil prokaryotes interact with soil fungi in bulk soil (see chapter 8 in this volume) and rhizospheres (see chapter 9 in this volume) and with protozoa (see chapter 11 in this volume). For the latter, they serve mainly as a nutrient source but they can also develop close symbiotic relationships with some of them.¹⁵ Soil prokaryotes can also be found in more highly evolved animals (e.g., nematodes, insects, or earthworms). They participate in digestion processes as gut inhabitants, but they can also occur in other regions of the body. The types of interactions between prokaryotes and eukaryotes in soil are manifold and they range from pathogenicity to symbiosis.

5.2 THE PROBLEM OF DIVERSITY MEASUREMENTS OF SOIL PROKARYOTES

The determination of “diversity” requires information on the number of different species (“richness”) and the number of individuals within each species (“evenness”). For “richness,” clear, reproducible assignments of individual organisms to species are needed and for “evenness” it is important to have a reliable quantification method.^{53,59} Both parameters, richness and evenness, however, cannot be unequivocally determined for prokaryotes in soil samples.⁸³

The species concept, which is the basis for any biodiversity assessment of eukaryotes, is not directly applicable to the prokaryotes, as prokaryotes lack the existence of groups that are reproductively isolated from each other. Even though there are clear definitions of what a prokaryotic species is, these definitions are for practical rather than biological reasons.¹¹³ Type strains for most defined species are deposited in culture collections as reference organisms. Alternative approaches to define species today are debated in light of new insights gained from genome sequencing, bioinformatics, and molecular phylogeny (“phylogenomics”).^{3,8,14} For ecological studies, perfect

species description may not be needed and, thus, the problem of species definitions for environmental isolates can be circumvented. Instead, prokaryotic isolates can be differentiated with a variety of techniques, such as a metabolic profiling on microtitre plates¹⁰ or typing of specific marker-genes (e.g., those encoding for the small subunit ribosomal RNA [SSU rRNA])^{107,115,134,139} (fig. 5.1). However, it should be noted that these short-cut techniques can be used to define operational taxonomic units (OTUs) or for a rather rough phylogenetic placement of an organism, but not for a taxonomically valid species description.

Cultivation is the classic, and yet for a prokaryotic species definition, the almost exclusive pathway. A typical protocol to isolate a diverse bacterial community from soil would include (1) suspension of a soil sample in sterile phosphate solution, (2) a series of 1:10 dilutions in a sterile buffer or saline, (3) inoculation onto agar-plates with nutrients, and (4) incubation of the inoculated agar-plates for one to several days at a temperature above 20°C. Such a technique will result in colony growth and, assuming that each colony was generated from a single cell, the number of bacteria per g of soil can be calculated. Typically, numbers detected from an agricultural soil sample following such a protocol will range between 1 million and 100 million cells per g of soil. The choice of nutrient agar has a tremendous impact on the number and richness of bacterial types that

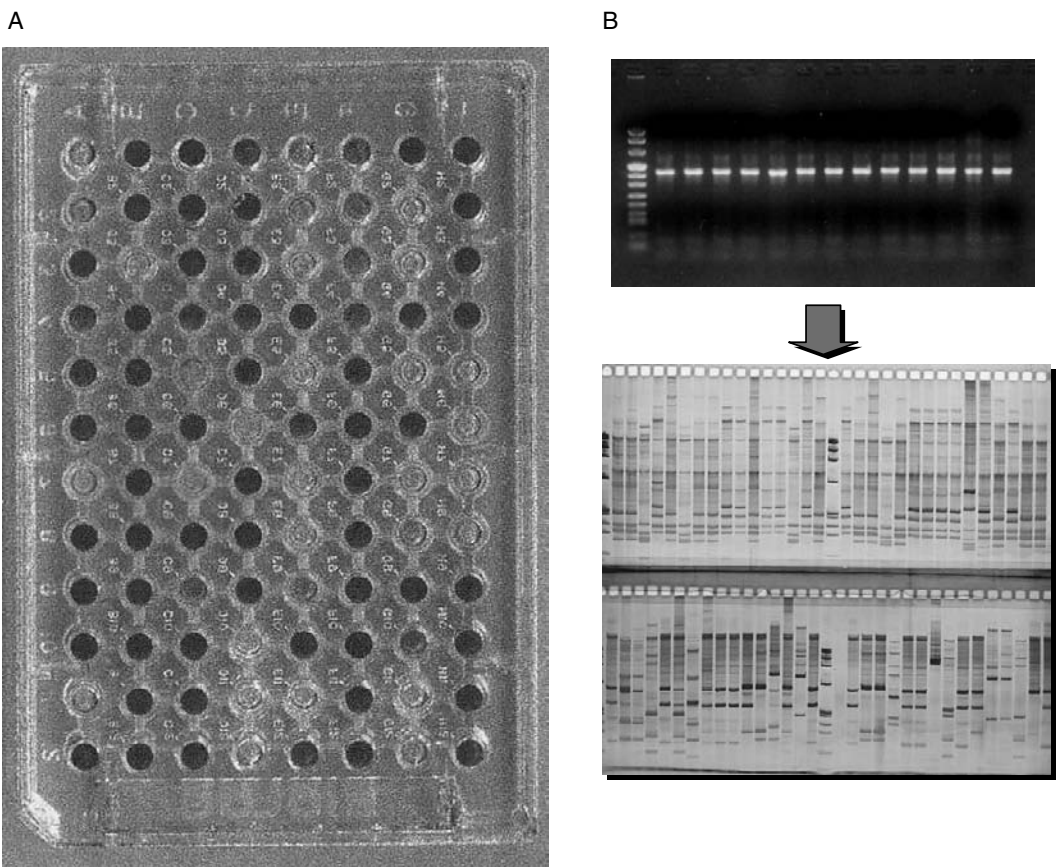


FIGURE 5.1 Techniques to differentiate soil bacteria according to their metabolic potential (A) or the structure of their 16S rRNA gene (B). Panel A shows a BiologGN[®] microtitre-plate that was inoculated with a bacterial pure culture. Each well contains a different carbon source and degraded carbon sources are indicated by a dark (red) color. In the upper part, Panel B, the PCR products of the 16S rRNA genes of different bacterial pure cultures of the same size (approx. 1,500 base pairs) are shown. In the lower part, two gels with the respective PCR products after digestion with two different restriction endonucleases are shown.

are isolated. This is a limitation, but it is also an opportunity, as many different soil bacteria can be isolated by varying culture media and incubation conditions.

The morphological diversity of colonies developing on agar surfaces is high and fascinating, and, based on the different colony types, it is possible to define and differentiate OTUs. Potentially, any OTU, if it maintains its ability to grow under laboratory conditions—which is not always the case—can be identified down to the species level. But colony morphology is not a reliable indicator, because some bacterial species have more than a single colony type and, on the other hand, many other species have colony types that are highly similar or the same. In addition, cultivation on selective agar also underestimates the genetic potential of the culturable bacteria.¹²⁰ In many applied studies, it may be tempting to use selective cultivation (e.g., to determine the level of mercury resistance in a polluted soil), but limitations such as growth inhibition upon direct selective cultivation should be kept in mind. Even more, as we know today that only a minority of soil bacteria can be detected with cultivation, it is obvious that with a rather unspecific, generalized cultivation approach, only dramatic changes in the total bacterial community would become detectable.^{73,85,126} By using directly extracted soil DNA and DNA-melting and reassociation kinetics, it was estimated that a single gram of soil may contain more than 4,000 different genomes and that classical plating techniques would underestimate the actual diversity by a factor of 200 (!).¹²⁵ In fact, recent estimates based on modeling bacterial diversity indicate that a gram of soil may contain 6,400 to 38,000 species,²⁶ and, thus, the limitations of cultivation for describing the prokaryotic diversity in soil is probably even greater.

5.3 ACCESSING THE NONCULTURABLE MAJORITY OF SOIL PROKARYOTES

The analysis of nucleic acids, DNA and RNA, directly extracted from environmental samples has provided the raw material that allows the characterization of the prokaryotic diversity in soil and other environmental substrates. Since the work of Pace and colleagues in the second half of the 1980s, the SSU rRNA or its corresponding gene was found to be the key molecule to investigate further the identity and diversity of the not-yet-cultured prokaryotes.^{84,86–88} The rRNA is an essential component of ribosomes, which are the protein “factories” in all living prokaryotic and eukaryotic cells. As the SSU rRNA gene is normally not transferred between different prokaryotes at evolutionary scales, it is a marker for the identity of an organism and its phylogenetic relation to other organisms (e.g., to type-culture strains). Comparisons of all known SSU rRNA gene sequences show that the gene is composed of conserved regions that are similar or identical in different organisms, and of nine variable or hypervariable regions scattered between. The variable regions accumulate mutations and, thus, they can be used as “molecular clocks,” meaning that the degree of differences in these regions between two organisms correlates with their phylogenetic distance.^{46,47,99}

The conserved regions, on the other hand, can be used as primer binding sites in the polymerase chain reaction (PCR). PCR is an *in vitro* reaction that allows specific amplification of selected DNA-molecules by a directed synthesis of the complementary strands of the DNA double-helix. Primers are oligonucleotides, which are complementary to specific regions in the DNA-molecule at the beginning and end of the selected region for PCR amplification. As some regions in the SSU rRNA genes are highly conserved, primers will also bind to SSU rRNA genes of yet uncharacterized organisms. To date, SSU rRNA gene sequences of more than 210,000 prokaryotes are known and accessible through public databases (e.g., EMBL [www.ebi.ac.uk], Genbank [www.ncbi.nlm.nih.gov], or RDP [rdp.cme.msu.edu]). This enormous sequence information allows the design of primers that specifically amplify SSU rRNA genes of different phylogenetic groups. The primer specificities are a big advantage of the PCR SSU rRNA-based studies. Depending on primer selection, the

resolution of amplified genes can be adjusted, for example, to the level of a phylum or subphylum, or, more sensitively, to detect and differentiate prokaryotes at the level of families or genera.

In order to study the prokaryotic diversity in soil, DNA can be extracted with different methods that are either “direct,” by lysing the cells in the soil matrix before DNA extraction, or “cell-based,” by first extracting intact cells, purifying them from soil contaminants, and then following a common protocol for extracting DNA from pure culture bacteria.¹³⁰ Direct methods have the disadvantage that they first generate DNA highly contaminated with other components, mainly humic acids. As humic acids are efficient inhibitors of the PCR, they need to be eliminated first.¹²¹ To date, however, a variety of straightforward methods, including a selection of commercially available kits, can be used to purify DNA from humic acids.

A typical nucleic acid-based approach to characterize the diversity of prokaryotes in a given soil sample would imply the following steps: (1) extraction and purification of total (genomic) DNA from soil, (2) PCR amplification of the almost complete SSU rRNA genes, (3) cloning of the PCR products in *Escherichia coli* in order to specifically amplify single PCR products, (4) screening of transformed *E. coli* strains for different PCR products (e.g., by ARDRA) (see fig. 5.1), and (5) sequencing of these cloned fragments. This protocol is followed by a bioinformatic data analysis including sequence alignments and annotations, identification of closest relatives from databases, and possibly more detailed phylogenetic analyses, as can be done, for example, with the ARB program package.⁶⁹ This methodological approach has been applied in an increasing number of studies on soil biodiversity during the last decade. It is through the achievement of these studies that we today have become aware of the major prokaryotic groups that contribute to the biodiversity in soils. The described methodological approach can be modified by replacing the time-consuming cloning and sequencing approach with a genetic profiling technique, such as DGGE (denaturing gradient gel electrophoresis), TRFLP (terminal restriction fragment length polymorphism), or SSCP (single-strand conformation polymorphism) (fig. 5.2).^{31,68,79} The attractiveness of using such techniques is that diversity patterns of different samples can be directly compared with each other, prior to a more detailed analysis, which would include the identification of common motifs or differences by DNA sequencing.

Despite their doubtless value, PCR-based methods also have limitations, as the diversity of amplified products may not accurately reflect the diversity of the original sample from which DNA was extracted. Prokaryotes can have different copy numbers of SSU rRNA genes and, within a single organism, these genes can even differ from each other, possibly in some cases as a result of gene duplication or horizontal gene transfer.^{2,127} In addition, the PCR reaction itself can generate biases (e.g., due to preferential amplification of some sequences from the original template mixture).^{118,135}

5.4 THE DOMINANT PROKARYOTIC GROUPS IN SOIL

Molecular analyses of different agricultural soils from the temperate climatic zone have indicated that most bacteria can be attributed to one of eight major phylogenetic groups.¹⁷ These groups are the *Proteobacteria* phylum with the classes *Alpha*-, *Beta*-, and *Gammaproteobacteria*, and the phyla *Actinobacteria*, *Bacteroidetes*, *Planctomycetales*, *Verrucomicrobia*, and *Acidobacteria* (named according to Bergey’s Manual Trust; www.cme.msu.edu/bergeys).¹⁸ It should be noted that from some of these groups (e.g., the *Proteobacteria* or the *Actinobacteria*), many different bacteria can also be recovered from soil samples with classical cultivation techniques, but others are normally missed. For the cultivated prokaryotes, laboratory studies can be applied to characterize their genetic equipment and physiological activity in more detail, and, thus, fairly good ideas about their potential ecological importance can be deduced in many cases. The ecological importance of the “not yet culturable” prokaryotes, evidently, is more cryptic.

The *Proteobacteria* are a physiologically and morphologically diverse group.^{47,145} *Alphaproteobacteria* include many bacteria that interact with eukaryotic hosts. Interactions with plants comprise the colonization of the rhizosphere, and the establishment of pathogenic or symbiotic relationships.

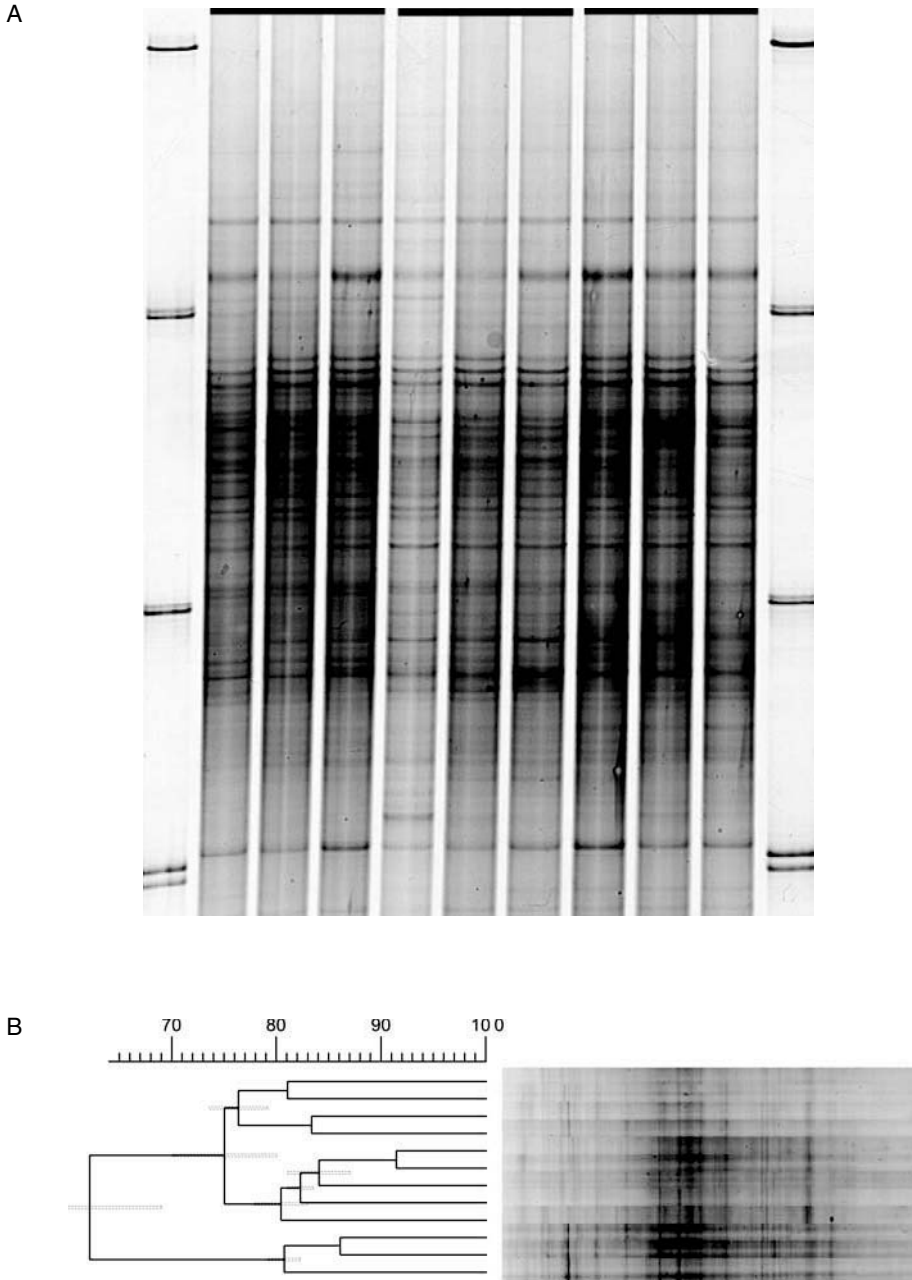


FIGURE 5.2 Example for a genetic profiling technique to study the bacterial community analyses on the basis of PCR-amplified bacterial SSU rRNA genes; here shown for consortia extracted from a rhizosphere soil. (A) SSCP-profiles of consortia from three individual plants, each represented by three replicates. The profiles are bordered by SSCP-standard lanes. (B) Digital image and hierarchical cluster analysis of profiles in order to characterize the similarity of patterns. Figure modified from Schmalenberger and Tebbe¹⁰⁶

Prominent examples are the crown gall diseases caused by *Rhizobium tumefaciens* (formerly *Agrobacterium tumefaciens*) and the formation of nitrogen-fixing root nodules in legumes by different members of the *Rhizobium* group (see chapter 12 in this volume).⁷¹ *Alphaproteobacteria* also include bacteria that infect soil-inhabiting invertebrates (e.g., *Wolbachia* of Collembola [springtails]).²⁸ Other

soil inhabiting *Alphaproteobacteria*, such as *Methylobacter* sp., aerobically utilize methane or other C1-compounds as carbon and energy sources.^{20,64,66} The *Beta*- and *Gammaproteobacteria* include many relatively fast-growing organisms that can be highly abundant in different soils—typical representatives include the genera *Burkholderia*, *Comamonas*, *Variovorax* (all *Betaproteobacteria*), and *Stenotrophomonas*, or *Pseudomonas* (both *Gammaproteobacteria*).

The *Actinobacteria* are Gram-positive bacteria and many of them are considered typical soil organisms.^{45,55,100} They are metabolically versatile, and many of them are spore-forming and can thus resist drought periods. The most prominent and abundant members include *Rhodococcus*, *Frankia* (induces molecular nitrogen-fixing root nodules of trees), and many species of the genus *Streptomyces*. *Streptomyces* are typical inhabitants of litter layers and they produce secondary metabolites (i.e., different antibiotics and geosmin, a compound that can be responsible for the earthy smell of a soil).⁴⁸ For both groups, *Proteobacteria* and *Actinobacteria*, the majority of the SSU rRNA sequences in the databases, as analyzed in the year 1998, were represented by cultivated isolates.⁵⁸

In contrast, only a few bacterial species have yet been cultivated and characterized from *Planctomycetes*, *Verrucomicrobia*, and *Acidobacteria*. Thus, only little is known about their genetic potential, physiological properties, or ecological adaptations, despite their obvious quantitative importance in soil.^{34,36,58} To date, only two isolates of the *Planctomycetes* have been obtained by cultivation from soil (i.e., one species related to the genus *Gemmata* and one to *Isosphaera*).¹³⁷ Other isolates were obtained from wastewater treatment plants or the marine environment. Interestingly, planctomycetes also include bacteria that are capable of oxidizing ammonium under anaerobic conditions in the presence of nitrite (“Anammox bacteria”).¹¹⁷ Whether these or similar bacteria are also active in anaerobic niches in soil is an open question.

The phylum *Verrucomicrobia* is only represented by a few cultivated species and it is unclear how representative they are for this large group. Some isolates from rice field soil are characterized by an anaerobic metabolism, and some of them, as well as other *Verrucomicrobia*, have a very small cell size (“ultramicrobacteria”).^{22,60} Other members are nonmotile with cellular appendices, like *Verrucomicrobium spinosum*¹⁰³ or *Prostheco bacter*.⁵⁰ The ecological range within this group seems to be large, as recently some species of the genus *Xiphinematobacter* have been characterized as obligatory endosymbionts in nematodes.^{132,133}

Acidobacteria, despite their high abundance in many soils,^{6,35} are also represented by only a few cultivated genera (i.e., *Acidobacterium*, *Geothrix*, and *Holophaga*). *Acidobacterium* is a moderately acidophilic heterotrophic organism⁶⁵ *Geothrix fermentans* is an Fe(III)-reducing bacterium that has been isolated from a hydrocarbon-contaminated aquifer²⁵; *Holophaga foetida* was isolated from anaerobic mud and can degrade aromatic compounds.⁶⁷ Recently, an approach to obtain more information about *Acidobacteria* from soil was conducted by cloning larger fragments of genomic DNA from a noncultivated organism. The results provided further support for the high abundance and diversity of *Acidobacteria* in soil.⁹⁷ In the same study, genomic analyses indicated that *Acidobacteria* had also acquired genes from other bacteria.

Candidate divisions are those phylogenetic branches that are not yet represented by cultivated isolates. As indicated by the SSUrRNA sequences recovered without cultivation from environmental samples, some of these groups are also abundant in soil (e.g., OP10, OP11, TM6, TM7, or WS6).^{11,23,32,33,58,102} Their ecological importance is not known, but targeted enrichment cultures and direct genomic cloning from soil DNA could be a feasible way to give rise to the first isolates in the near future and, thus, will help to elucidate their function in soil. Another useful approach for studying the ecological importance of these soil prokaryotes is to follow their population dynamics in response to environmental changes (seasonal effects) or their spatial distribution. Methodologically, this can be done by measuring the relative abundance of ribosomal rRNA with specific probes or genetic profiles.^{17,82} Figure 5.3 shows an example in which the bacterial diversity inhabiting the rhizosphere of chamomile was studied with a community-fingerprinting technique using primers with adjusted specificities of different bacterial groups. The dominant bands, seen with the *Bacteria*-specificity, are

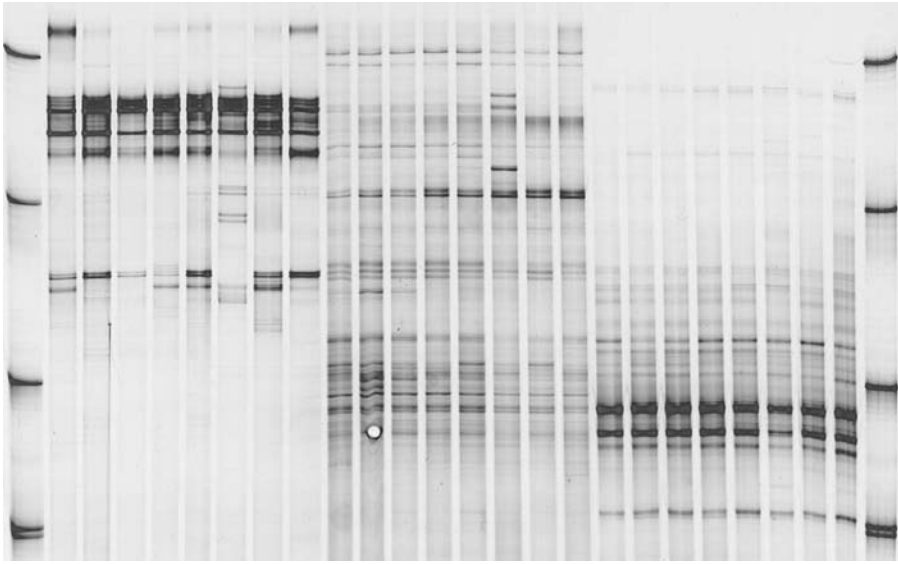


FIGURE 5.3 SSCP-profiles of the bacterial community inhabiting rhizosphere soil of chamomile (*Matricaria chaemomilla*). The gel shows the bacterial diversity as seen by PCR-amplified SSU rRNA genes using primers with different specificities, each in eight replicates for the genus *Pseudomonas* (left lanes), for *Eubacteria* (center lanes), and for *Alphaproteobacteria* (right lanes). The selected technique (“nested PCR”) allows a direct comparison of the patterns with each other. Lanes on the far right and far left side of the gel contain SSCP-markers. Figure from Dohrmann and Tebbe,³¹ 2004, with permission.

not well represented by the patterns generated for *Pseudomonas* or *Alphaproteobacteria*, indicating that other bacteria rather than these easily culturable groups were dominant.

In addition to the previously mentioned groups, members of the *Bacillus* and *Clostridium* group (Phylum: *Firmicutes*) can frequently be detected by both cultivation-based and cultivation-independent analyses of soil samples, and sometimes their abundance is very high.³⁷ This group is characterized by their capacity to form endospores and survive for very long periods of time without the need for external carbon or energy sources. A common motif of many of these bacteria is the capacity for rapid growth when relatively simple carbon sources become available. The substrates can be supplied by roots in the rhizosphere,¹⁰⁸ by digestive processes in the gut of invertebrates^{27,43,61,76} or by plant residues, the latter also indicated by the rapid development of thermophilic *Bacillus* species in composting processes.⁹⁰ Some members of the genus *Bacillus* have developed strategies to multiply in different host organisms and become pathogens (e.g., *B. thuringiensis* strains in certain insects⁹⁸ or *B. anthracis* in mammals⁷⁵).

The discrepancy between the diversity of cultivated isolates and the actual diversity that is detected by molecular methods in soil is even higher for the Domain *Archaea* than for *Bacteria*. Cultivated soil suspensions incubated on nutrient agar under aerobic conditions will rarely show archaeal colonies. Most *Archaea* have been isolated from extreme environments or specific ecological niches. All cultivated members belong to one of two phylogenetic lineages (Phyla) (i.e., *Crenarchaeota* and *Euryarchaeota*). On the other hand, the cultured members of the Phylum *Euryarchaeota* are either extreme halophiles, thermophiles, sulfur- or sulfate-reducers, or methanogens. Methanogenic *Euryarchaeota* have been isolated from soils with high methane production rates (e.g., rice fields or natural wetland soils) or from the anaerobic layers in landfill sites.^{38,51,66,77,142} In addition, *Euryarchaeota* can colonize the digestive system of soil invertebrates, such as soil-feeding termites, where they convert the metabolites acetic acid and molecular hydrogen to methane.⁴⁰

The properties of cultivated *Archaea* suggest that they are highly adapted specialists, but the detection of SSU rRNA genes of not-yet-cultivated *Archaea* in many different soils contradicts this idea. Many different archaeal SSU rRNA genes can be found in both anaerobic and aerobic soil environments.⁹ The wide abundance of *Crenarchaeota* sequences in soils from the temperate climatic regions, in which they occur in bulk soils and rhizospheres, suggest the existence of nonthermophilic members in this group.^{19,62,80,109,110} Euryarchaeal SSU rRNA sequences most closely related to the order *Thermoplasmatales*, which comprises extreme thermophiles, have been detected in the aerated zone of a forest soil in the alpine region.⁸⁹ Molecular analyses of SSU rRNA genes from environmental DNA also show the existence of more than the two Phyla already described for archaea, and several of them may contribute to the biodiversity found in soils.^{7,9,30}

5.5 THE IMPACT OF AGRICULTURE ON SOIL PROKARYOTIC DIVERSITY: A VIEW ON STRUCTURE

As indicated earlier, the biodiversity of soil prokaryotes coupled to their metabolic versatility is one of the key factors contributing to the sustainability of soils in agroecosystems. It is therefore necessary to consider soil prokaryotic biodiversity as an important parameter for evaluating the ecological impact of agriculture on soils. As the definition of species and groups for prokaryotes is more arbitrary than ecologically meaningful, there is no fixed scale on which such impact studies should be conducted. As a rough but possibly very meaningful parameter, the total biomass of, for example, bacteria may be compared to that of fungi or archaea. More sensitive parameters would include the characterization of groups or of species or, furthermore, the diversity of strains within a defined species. It is only rarely possible to link a certain species diversity with an agricultural function, but a prominent example are the legume-nodulating rhizobia. Different species from this group can cause root nodule formation in different legume species and strains can be differentiated within a single *Rhizobium* species, for example, by genetic typing of bacteria in nodules (see also chapter 10 in this volume). Another example is probably the ammonium-oxidizing bacteria in soils, which respond with changes in their diversity to different agricultural management conditions^{16,72,93} (see also Sections 6 and 7). However, for most soil prokaryotes, such direct links between a species or group of organisms and an agriculturally relevant property cannot be made.

In a case study, the soil microbial diversity of conventionally managed fields was compared to fields under short-term and long-term organic farming, respectively.¹⁰⁴ A hierarchical approach was applied, meaning that the samples were compared in terms of their microbial biomass (bacteria, archaea, fungi), their species diversity, and by the diversity of ecotypes of one selected species (fig. 5.4). It turned out that the microbial biomass of the investigated field plots from the different treatments did not differ from each other. In contrast, significant changes of the microbial community were detected on all other taxonomic levels that were analyzed. On the domain level (bacteria, archaea, and fungi) shifts in the structure of the microbial community became detectable within two years after changing the farming practice. With increasing time, these differences became even more evident. The main shifts were found in Gram-negative bacteria, which decreased the longer the organic farming practices were imposed. These changes could also be seen at the species level. Whereas a high abundance of Gram-negative bacteria such as *Burkholderia* and *Pseudomonas* was detected on plots treated by conventional farming, plots that had been farmed organically for a long time were dominated by *Actinobacteria*. However, the overall diversity based on the calculation of the Shannon Index, which considers the factors “species richness” and “evenness” (see Section 2), was similar and not significantly different between the treatments. Interestingly, the fungal diversity was mostly unaffected by the type of soil management. As an indicator of strain (ecotype) diversity, the population of bacteria belonging to the group *Ochrobactrum* was studied. Although the overall diversity based on the Shannon Index was unaffected, a clear shift in the types of strains that occurred could be detected. The strains that were stimulated by organic farming had the ability to

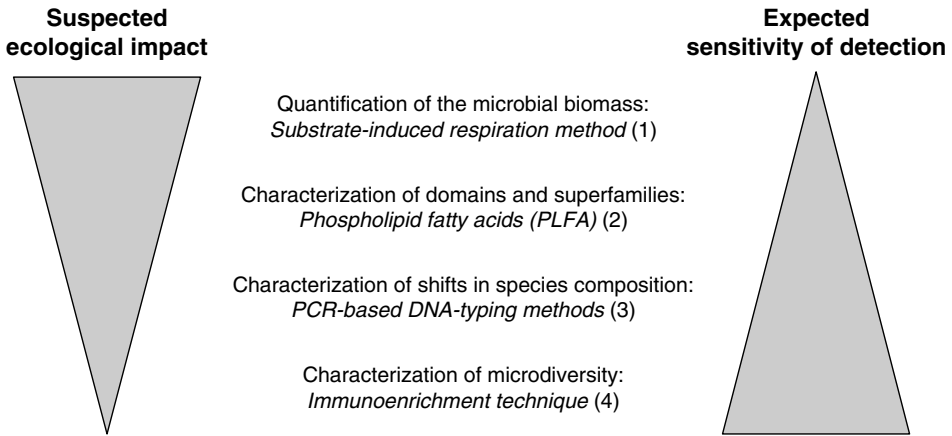


FIGURE 5.4 Hierarchical approach to study the changes in the composition of soil microbial communities, that is, as a response to agricultural management practices. Numbers in parentheses indicate representative references for each of the selected parameters; (1)⁴, (2)¹⁴⁶, (3)¹²⁹, (4).¹⁰⁴

utilize a wider range of carbon sources than those from the conventional site, thus reflecting the higher diversity of substrates that may be present in an organically managed soil.

5.6 THE LIMITATIONS OF STRUCTURAL DIVERSITY MEASUREMENTS: THE NEED FOR FUNCTIONAL DIVERSITY MEASUREMENTS

In ecological studies, a catalogue of the different species in a given environment cannot be a goal, but only a tool, helping to understand interactions and dependencies of different organisms on each other or on environmental parameters. For higher organisms (e.g., members of the soil mesofauna), a species or a certain taxonomic group can often be connected to functional parameters, for example, epiphytic collembola enhance the decomposition of organic material in the litter layer or earthworms transport organic substrates into deeper soil layers and increase the number of soil macropores. With soil prokaryotes, however, things are different: In most cases direct links between a species and an ecological function cannot be made. Most ecological functions in soil are provided by many different species from different phylogenetic groups. Cellulose degradation, for example, is an important process in the carbon cycle, and bacteria with cellulases can be found in many phyla (e.g., *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, or *Firmicutes*).¹²⁸ The same is true for denitrifying or nitrogen-fixing bacteria.¹⁴⁶ The soil-inhabiting ammonium-oxidizing bacteria, however, are probably an exception. Only members of the two genera *Nitrosospora* and *Nitrosomonas*, both belonging to the *Betaproteobacteria*, have been found as ammonium-oxidizers in the terrestrial ecosystem to date.^{1,5,78,95} It may turn out in the future, though, that other microorganisms (e.g., from the not-yet-cultivated groups), are also capable in oxidizing ammonium in soil.

A common feature of microbiologically mediated functions in soil is that a number of different organisms contribute to a specific function, for example, cellulose degradation, denitrification, or the production of plant-growth promoting compounds. These functional redundancies in soil microbial communities can, at least partially, be explained by the heterogeneity of soil with its different microhabitat conditions in a small space.^{39,42,101,138} At the evolutionary scale, this functional redundancy means that either the same functions evolved several times, independently of each other; that they were developed very early; or that the genetic information has frequently been transferred between organisms. Due to the importance of lateral gene flow for the evolution of prokaryota, which recently became evident through comparative analyses of genome sequences, the first two processes

cannot clearly be separated.^{12,29,91,129} In any case, the result of prokaryotic evolution with less specific boundaries than biological species means that species and functions are not as closely linked in the prokaryotic world as they are in eukaryotes.

Horizontal gene transfer of mobile genetic elements is an important factor that may decouple structure and function in bacterial communities. Several functionally important genes (e.g., those that encode for the degradation of different xenobiotic compounds), are located on mobile genetic elements.¹¹² Genes that are located on conjugative plasmids have a particularly high potential for transfer to other bacteria. Such transfer events can become evident in populations under selective conditions. Conjugative gene transfer in soil is not a rare event^{24,124}. In fact, several studies have shown stimulated transfer under the influence of nutrients⁴⁴ or in specific niches (e.g., in rhizospheres¹¹¹ or the gut of invertebrates^{54,122}). Thus, depending on the specific conditions in a microhabitat and the force of selection, a specific function may be provided by only one or a few species or by many. On the other hand, due to plasmid segregation, a single species even in the same ecological niche may or may not harbor a plasmid with a specific function.

Instead of detecting the structural diversity (e.g., by means of 16S rRNA gene analyses), the concept of functional diversity has recently been emphasized as an alternative that can be highly relevant for ecological studies.⁶³ The focus of functional diversity measurements is a specific function provided by soil microorganisms, for example, nitrogen fixation, denitrification, degradation of a specific compound, or production of a plant growth promoting factor (also see chapter 12 in this volume). As discussed before, in most instances, a function will be provided by different members of the soil prokaryotic community. The question, however, is which member provides these functions, how redundant is the function in a community, and can the same function be fulfilled by completely different organisms in similar ecological niches. The answer to this question is linked to the problem of whether biodiversity measurements of soil prokaryotes or selected “species” can serve as indicators for the quality of a soil, or more generally to the “value” of prokaryotic diversity.^{83,136} Functional diversity measurements may offer more meaningful and more sensitive approaches for detecting the effect of changing environmental factors on the soil prokaryotic community.¹⁴⁴ Functional diversity measurements may thus be better suited for detecting effects of different agricultural practices (e.g., organic vs. conventional farming, tillage vs. nontillage, or genetically engineered crops vs. nonengineered counterparts).

5.7 THE IMPACT OF AGRICULTURE ON SOIL PROKARYOTIC DIVERSITY: A VIEW ON FUNCTIONAL DIVERSITY IN THE NITROGEN CYCLE

In order to tag and detect functional genes in DNA directly extracted from soil, solid background knowledge about the genetic diversity of a selected function is needed, especially because primers need to be designed and optimized that hybridize to conserved regions of such targeted genes. As more and more sequences become available from the public databases, it can be assumed that this background knowledge will increase enormously in the near future, thus allowing the tagging of many different, functionally important genes of the soil prokaryotic community.

To date, a major focus in studying the functional diversity of soil prokaryotes has been the analysis of functions connected to the biologically mediated nitrogen cycle. The nitrogen cycle includes ecologically important steps, such as nitrification, denitrification, or nitrogen fixation. Nitrification converts ammonium to nitrate under aerobic conditions. Nitrate is the most important nitrogen source for plants and thus, in agricultural systems, nitrification is directly connected to soil fertility and plant growth. Denitrification is the conversion of nitrate to molecular nitrogen (N_2 gas), which is a process that is mainly conducted under anaerobic conditions by nitrate-respiring microorganisms. Both processes, nitrification and denitrification, also produce nitrogenous greenhouse gases (i.e., N_2O). Finally, nitrogen fixation is the only biological process by which the large pool

of N_2 in our atmosphere is converted into biologically available ammonium. For all of these systems, as well as for others, it is of interest to characterize the diversity of organisms that carry out these functions. Functional diversity measurements can first tell which group of organisms represents a certain function in a specific niche or under specific conditions. This information can then be used, for example, to understand in more detail how microbial communities adapt to changing environmental conditions, which physiological properties of prokaryotes may be important,⁹⁵ or how certain functions are buffered under conditions of stress.

The ammonium-oxidizing bacteria convert ammonia to nitrite in a two-step process. The first step is the conversion of ammonia to hydroxylamine catalyzed by the enzyme ammonium mono-oxygenase, the latter being encoded by the *amoA* gene.⁵⁶ In fact, it could be shown that the addition of N-fertilizers can have significant effects on diversity and heterogeneity of the *amoA* gene pool.¹³⁸ The diversity of *amoA* genes was higher in unimproved soils than in soils to which nitrogen was added. Replicate soil samples of unimproved soil demonstrated significant spatial heterogeneities, detectable as clusters of the *amoA* genes in phylogenetic analyses. The differences were correlated with a higher diversity of ammonium concentrations and pH, due to the spatial heterogeneities in the unimproved soils, as the heterogeneity in the fertilized soils was lower. The effects of N-fertilizer on the abundance of *amoA* genes could be demonstrated in a study just three days after the application of a nitrogen fertilizer.⁷⁴ However, the size of the gene pool of *amoA* was not correlated to the rates of nitrification, which were determined in parallel by a ^{15}N isotopic pool dilution assay. Although 10^4 to 10^5 gene copies *amoA* per g of soil were present before the application of the fertilizer, the actual nitrification rates were low. Shortly after the nitrogen application, the gene pool was unaffected. However, the rates of nitrification increased immediately. Subsequently, ammonium concentrations and nitrification rates decreased, while the pool-size of *amoA* genes increased in plots with fertilizers. These results clearly indicated that fertilization had an immediate effect on the transcription of *amoA* and, later, selected for differently adapted subpopulations of the ammonium-oxidizing bacteria.

In contrast to ammonium oxidation, denitrification can be conducted by soil prokaryotes from many different phylogenetic groups. Most denitrifying bacteria preferentially respire O_2 but in its absence, nitrate can be utilized as an electron acceptor. Denitrification is stimulated in wet soils in which oxygen is limited but nitrate is available.^{41,83} Although many denitrifying bacteria completely reduce NO_3^- to N_2 , denitrification, in principle, consists of three independent steps, that is, (1) $NO_3^- \rightarrow NO_2^-$, (2) $NO_2^- \rightarrow NO \rightarrow N_2O$, and (3) $N_2O \rightarrow N_2$.¹⁴⁷ The nitrite reductase gene (*nirK*), which encodes for the key enzymes of reaction (2), could be detected and quantified independently of cultivation by real-time PCR in different soil samples at 10^5 to more than 10^6 copies per g.⁵² Sequencing and phylogenetic analysis of amplified products indicated that most of the *nirK* genes originated from yet-uncultivated soil denitrifying soil bacteria. In other studies *nirK* was detected in soil samples at concentrations of 10^8 to 10^9 copies per g and the numbers appeared to be correlated with the ammonium content in the soils.⁹⁶ Alternatively, or in addition to *nirK*, some studies have analyzed the abundance and diversity of the *nirS* or *nosZ* genes as indicators for denitrifying bacteria.^{13,94,123} Like *nirK*, the *nirS* gene also encodes for nitrite reductase, while the *nosZ* gene encodes for the nitrous oxide reductase. Another genetic marker for denitrification is the gene that encodes for the membrane-bound nitrate reductase, *narG*. In a number of recent studies it could be shown that all of these functional genetic markers can be useful for detecting the effect of agricultural management practices on soil.^{21,92,116,123}

Nitrogen fixation is performed in most soil prokaryotes by the nitrogenase protein complex, which catalyzes the reduction of N_2 to ammonium. Archaea and Bacteria from several phylogenetic groups have been shown to be capable of fixing nitrogen, among them symbiotic bacteria (i.e., the previously mentioned rhizobia) and nonsymbionts (i.e., methanotrophs, cyanobacteria, and many other soil bacteria).¹⁴⁶ Nitrogenase is an evolutionarily relatively conserved protein complex, composed of two multisubunit metallo-proteins.^{57,146} At the genetic level, the nitrogenase genes are

located in complex operons. One of the most conserved regions in this operon is the *nifH* gene. Several studies have in fact demonstrated that this gene can be used as a target sequence to study the functional diversity of nitrogen-fixing bacterial communities in different types of soils and rhizospheres.^{49,119,141}

5.8 CONCLUSIONS

In this chapter we have introduced the diversity of prokaryotes in soils and tried to emphasize the specificities that should be considered for using diversity measurements of prokaryotes in order to determine the impact of agricultural management practices or other factors on these major constituents of the soil microbiota. Undoubtedly, the majority of soil prokaryotes have not yet been cultivated and characterized in detail, and the importance of developing more sophisticated cultivation techniques to access this enormous genetic reservoir is evident. Nevertheless, the methodology for characterizing the structural and functional diversity of soil prokaryotes is already sufficiently well developed to collect meaningful data in field studies. Yet, the current methodology based on nucleic acid analyses of PCR-amplified products is not an endpoint in the development of molecular tools to study the soil prokaryotic diversity. As sequencing information improves our knowledge about the genetic diversity in soils and DNA detection methods become more sensitive, DNA-microarray technology will increasingly allow simultaneous investigation of many different genes and gene families from a single nucleic acid sample extracted from soil. In addition, it becomes feasible to distinguish active from inactive cells and to detect specific gene expressions, for example, by using recombinant marker genes, stable isotope probing, or highly sensitive quantifications of transcribed genes (mRNA) and their synthesized proteins. It is this combination of methods that will help further elucidate the contribution of prokaryotes to the maintenance of soil fertility and health.

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6 Microbial Diversity in the Rhizosphere: Highly Resolving Molecular Methodology to Study Plant-Beneficial Rhizosphere Bacteria

Anton Hartmann, Kornelia Smalla, and Jan Sørensen

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6.1 THE RHIZOSPHERE: A VERY SPECIAL SOIL HABITAT

In the plant root–soil interphase, exudates from the roots together with the roots themselves create a very unique microbial habitat, which is very different from the bulk soil that is not influenced by roots. The first definition of the rhizosphere—the soil compartment influenced by roots—was already made a hundred years ago by Lorenz Hiltner.⁶⁶ Dependent on plant species and age, a specific cocktail of low molecular weight (sugars, organic acids, amino acids, and vitamins) and polymeric compounds reaches the soil, amounting up to 20–30% of the total photoassimilation of the plant.^{113,121} Surely this attracts a high number of bacteria and fungi, which may be beneficial or pathogenic to the plant. In turn, a high competition for colonization of the rhizosphere is created, leading to mobilization of soil micronutrients, which may become limiting under the high nutrient demand in the rhizosphere.^{23,52} The soil harbors an extremely high diversity of indigenous microorganisms hidden within its complex structure (aggregates, pore structures, and water films). However, only a very small fraction of this entire genetic pool is active at a given time and space. Apart from the specific habitats like the intestine and gut of soil animals, surfaces of dead and living plant material are stimulating a specific subset of microbes to highly active colonization and interaction.¹⁸² The relevance of these plant–microbe interactions for plant health and nutrition has motivated scientists to study the diversity of rhizosphere microbes in detail. This has brought about major progress in understanding the relationship between structural and functional diversity of microbial communities in the rhizosphere and has lead to the identification and exploitation of new beneficial microorganisms as inoculants to stimulate or protect plant growth or to contribute to remediation of polluted soils. Combined with the availability of molecular biological techniques for microbial ecology in the last 15 years, single-cell or population-level studies of symbiotic nitrogen-fixing bacteria (e.g., rhizobia), diazotrophic plant-growth promoting bacteria (e.g., *Azospirillum* spp.), and biocontrol active bacteria (e.g., *Pseudomonas* spp.) have contributed significantly to the understanding of the structural and functional diversity of rhizosphere microbes.

A major limitation for studies of the population structure and function of either indigenous or inoculated microbes in soil was for long the lack of appropriate microscale and *in situ* technologies to differentiate specific populations and to observe their activity at the single-cell level. Over the past decade, however, a vast amount of new information on bacterial abundance and distribution and on limiting factors of growth in the soil environment has been collected. Hartmann et al.⁵⁹ and Chin-a-Woeng et al.²⁷ recently reviewed conventional and molecular *in situ* and *ex situ* methods used to characterize the structure and function of rhizosphere microbial communities with a complementary set of methods. Sørensen and Nybroe¹⁶⁵ recently summarized approaches based on classical and modern technologies to study indigenous or inoculated *Pseudomonas* spp. populations in bulk soil and rhizosphere. Sørensen et al.¹⁶⁴ further gave specific examples on the use of inoculated *Pseudomonas* strains (equipped with reporter genes) to provide information of limiting factors (oxygen, nitrogen, phosphorus, and iron availability) in bulk soil and rhizosphere environments.

This chapter summarizes the most important characteristics for the diversification of soil bacteria in the rhizosphere, including an introductory treatise of modern methodology to assess the diversity of the microbial composition and activity at population and community levels in soil. Special emphasis will be made on exemplifying diversity for important groups of rhizosphere bacteria, relating to nitrogen-fixing bacteria and to bacteria offering plant growth stimulation or protection against fungal pathogens.

6.2 METHODS TO STUDY MICROBIAL DIVERSITY IN SOIL AND RHIZOSPHERE

Traditionally, the diversity of microbes residing in soil and their responses to environmental variation has been studied either by plating or enrichment techniques or by the so-called “black box” methods such as determination of respiration rates, “microbial biomass” by, for example, substrate-induced respiration rates, C- and N-mineralization rates, or enzyme essays.¹²⁰ While these methods provide information on microbial and enzyme activities in soils and the rhizosphere,⁸⁰ they are not suited for microbial diversity analysis on the organismic level. Recent progress was made to miniaturize enzyme assays by using fluorogenic substrates and applying test conditions more closely related to soil and rhizosphere conditions.¹³⁹ These approaches will not be further discussed in this chapter.

6.2.1 CULTIVATION-BASED METHODS

The cultivation-based methods are obviously biased toward those bacteria or fungi that can form colonies on nutrient media. The observation that only a small proportion of bacteria can form colonies when traditional plating techniques are applied was early described as the “great plate anomaly” by Staley and Konopka.¹⁶⁶ Based on microscopic studies it was later estimated that approximately 0.3% of the total bacterial cells in soil are accessible to traditional cultivation.³ Recent studies in which diluted media and long incubation times were used actually resulted in a higher proportion of cultured bacteria, but 27% of the isolates belonged to bacterial classes and phyla, which are poorly represented by cultured isolates.^{75,79} The application of a whole set of different growth media also increased the representativity of the cultured bacteria in the rhizosphere.⁵⁹ Another problem of cultivation-based methods is that bacteria, which are usually culturable, can enter a state called “viable but nonculturable” (VBNC); again, these bacteria would not be accessible to traditional cultivation techniques.^{129, 145} Studies on culturable bacteria will not adequately reflect indigenous microbial diversity in soil. Culture-independent studies have the power to open new phylogenetic views of bacterial diversity in soils.^{69, 97, 123}

6.2.2 ANALYSIS OF THE TOTAL MICROBIAL COMMUNITY

In the following, we will provide an overview of some biomarker and recent molecular biological techniques to study diversity within indigenous microbial communities of soils and the rhizosphere.

6.2.2.1 Phenotypic Fingerprints: BIOLOG Substrate Utilization Patterns

BIOLOG GN-plates were originally developed for the identification of axenic cultures based on their ability to oxidize 95 different carbon sources. Garland et al.⁴⁷ applied BIOLOG plates to characterize total microbial communities. Microbial cell suspensions directly recovered from soil are inoculated into the wells of a 96-well plate containing the tetrazolium dye and either 95 different carbon sources in the case of BIOLOG GN, or three times 31 carbon sources in BIOLOG-Eco plates. The plates are incubated for an appropriate period of time, and the oxidative substrate utilization can be monitored by measuring reduction of the tetrazolium dye.

BIOLOG GN-plates have subsequently been used to assess substrate utilization patterns of many soil microbial communities due to the ease of application.⁷⁴ Numerous studies have shown that microbial communities produce habitat-specific and reproducible patterns of carbon utilization. Furthermore, the BIOLOG assay has been widely used to study temporal and spatial changes of soil microbial communities, or in response to environmental perturbations.^{55,64} Originally BIOLOG substrate utilization patterns were claimed to provide information on the functional diversity of soil microbial communities, but there has also been criticism. Hence, by analyzing the bacterial communities in different substrate wells by DGGE (see below) it became obvious that only a small proportion of bacteria, which are fast-growing and able to compete under the actual growth

conditions, contributed to the BIOLOG patterns.¹⁶⁰ BIOLOG is a rather simple and sensitive method to detect changes in soil microbial communities, which can also be quantified. However, the interpretation of the changes in substrate utilization patterns remains difficult.

6.2.2.2 Chemotypic Fingerprints: Phospholipid Fatty Acid (PLFA) Patterns

Phospholipid fatty acid (PLFA) patterns can be compared by multivariate statistics to indicate changes in microbial communities and have been used to obtain insights in how soil microbial communities respond to different stresses. The method is based on the determination of microbial membrane phospholipids extracted directly from the soil samples by gel chromatography. The chemical character of the linkages of the side chains with the glycerol backbone can serve as a taxonomic biomarker.¹⁹⁹ Although specific PLFAs can be used as indicators (e.g., 18:2 ω 6,9 as an indicator for fungal biomass in soil), the interpretation of PLFA patterns from soil remains a challenge. However, data bank analysis of PLFA patterns is available to facilitate the interpretation of the information retrieved from the highly resolving PLFA analysis (A. Gatteringer, GSF-Institute of Soil Ecology, Neuherberg, Germany).

Microbial biomass and total PLFA usually correlate to organic carbon and total nitrogen, indicating that these measures are good indicators of soil fertility.¹⁹⁶ PLFA analysis is suited as a routine method to assess broad-spectrum differences of soil communities in response to land use or pollutions, correlating well with changes in soil fertility.^{9, 196} Changes of PLFA patterns in soils after liming were temperature-dependent, with the most rapid changes observed at 30°C, while no changes could be seen at 5°C.¹³⁶ The determination of phospholipid etherlipid (PLEL) fatty acid patterns to characterize *Archaea* communities in soils is a rather new approach.⁴⁸ Monomethyl-branched alkanes were the most dominant ones of the total ether-linked hydrocarbons detected in soils, followed by unbranched and isoprenoid hydrocarbons; the latter have not been detected yet in archaeal isolates.

6.2.2.3 Genotypic (Genetic) Fingerprints (Ribosomal and Metabolic Genes)

The development of methods to analyze nucleic acid composition in environmental samples has opened a new dimension to study microbial communities (for review, see van Elsland et al.¹⁷⁹). The analysis of DNA can thus provide information on the structural diversity of indigenous soil populations or on their functional diversity (e.g., presence or absence of certain genes) or on the fate of introduced bacteria. However, the analysis of DNA does not allow conclusions on the active subpopulations in the bacterial or fungal community, or on specific gene expression. This information might be obtained from assays based on DNA synthesis (replication) or RNA synthesis (transcription). As several assays based on environmental DNA or RNA analysis have been developed recently, an introduction to the assays is made in the following.

6.2.2.3.1 Nucleic Acid (DNA/RNA) Extraction

Nucleic acid extraction from soil matrices remains a challenge, in particular for RNA. This is mirrored by the large number of publications on this subject.¹⁷⁹ None of the protocols are suitable for all kinds of soils, in particular the soils from contaminated sites. Only recently commercial kits became available for DNA extraction from soil; this has indeed been a major breakthrough for simplification and miniaturization of this crucial step for cultivation-independent analysis methods. DNA can be extracted either directly from the soil matrix or after prior recovery of the microbial fraction. The advantage of the direct nucleic acid extraction approach is that it is less time-consuming and that a much higher DNA yield is achieved; however, directly extracted DNA often contains considerable amounts of co-extracted substances such as humic acids, which interfere with the subsequent molecular analysis. The indirect DNA extraction approach might preferably be used when problematic soils (e.g., heavy metal polluted soils²²) are to be analyzed, or when large DNA

fragments from soil are cloned into BAC (bacterial artificial chromosome) vectors. Published protocols differ considerably with respect to the solutions used to break up soil colloids and dislodge surface-attached cells, which adhere by various bonding mechanisms.^{10, 179} A potential bias of the indirect method is a preferential dislodgement of cells that are less tightly bound to the soil surface. DNA recovered from microbial pellets is usually less contaminated with co-extracted humic acids and DNA of nonbacterial origin. The efficient disruption of the bacterial and fungal cell walls is crucial for the recovery of representative DNA but might result in DNA shearing. High-molecular-weight DNA is another important criterion when evaluating and comparing different protocols because sheared DNA can cause PCR artifacts and is not suitable for direct cloning of large DNA fragments.

In contrast to direct and indirect DNA extraction protocols, which are used almost routinely, methods for RNA extraction are less frequently applied and considerable efforts are needed to ensure the absence of RNases. The presence of humic acids and residual DNA in RNA extracts are critical for further molecular biological analysis.² Due to the short half-life of bacterial messenger RNA, an unbiased recovery of total RNA is a great methodological challenge. Different protocols aiming at the simultaneous extraction of RNA and DNA have recently been published.^{54, 72} The efficient removal of humic substances and residual DNA without partial degradation of the RNA is crucial for the reliable use of RNA for microbial community analysis. Most recently, microbial RNA isolated from plant litter of forest soils was successfully used to investigate active microbial communities.⁴

6.2.2.3.2 PCR and PCR Primers

The most frequently used phylogenetic markers are the genes coding for the ribosomal RNA. The rDNA molecule offers great potential as a phylogenetic marker because it is universal to all forms of life, it is structurally and functionally conserved and it contains regions with different degrees of relatedness. Another considerable advantage is the rapidly growing database of ribosomal rDNA sequences and the analysis of rDNA sequences thus provides a strong phylogenetic framework. A disadvantage for its use in cultivation-independent microbiology is that bacteria possess different numbers of ribosomal DNA operons, which might reflect different ecological strategies of bacteria⁸³; also, sequence heterogeneity of the different operons might occur.^{127, 186} The primers targeting the ribosomal rRNA genes can be designed in such a way that only ribosomal rRNA fragments of bacteria, archaea, or fungi can be amplified from DNA extracted directly from soil. When the PCR amplicons are analyzed with one of the above-mentioned fingerprinting techniques, a display of the dominant bacterial, archaeal, or fungal populations is obtained. With the use of taxon-specific primers, the complexity of the soil community patterns could be reduced.^{16, 53, 62} Such a reduction of the complexity might be required in particular for soils with high numbers of equally abundant populations. Hence, Dunbar et al.⁴² reported that for highly complex communities the method appears to be unable to provide a classical measure of soil diversity. Furthermore, the application of group-specific primers enables to study less abundant populations. The advantage of ribosomal RNA gene-based fingerprints is that these can be compared to demonstrate differences between different soils or treatments and that information on the phylogenetic affiliation of dominant or differentiating populations is made available.

For specific functions of bacteria, diversity studies can also be based on primers for functional genes such as the ammonia monooxygenase gene (*amoA*).¹⁹⁰ the methane monooxygenase gene (*pmoA*).^{29, 92} the nitrogenase reductase gene (*nifH*).^{172, 192, 200} or the ribulose-1,5-bisphosphate decarboxylase/oxygenase gene (*cbbL*).^{119, 157} In addition, functional genes coding for chitinases or for enzymes involved in nitrification and denitrification processes has been studied by PCR amplification of the respective genes from community DNA, followed by cloning, restriction analysis (RFLP), and sequencing^{21, 25, 111, 138, 169} (see chapter 5, in this volume).

6.2.2.3.3 *PCR-Amplification of 16S rDNA, Fingerprint, and Clone Bank Analyses*

Multiple sample analysis is essential when spatial and temporal variability of soil microbes are evaluated in relation to environmental factors or to perturbation. Most appropriate are here the fingerprinting techniques like denaturing or temperature gradient gel electrophoresis (DGGE or TGGE),^{63,80,106,112,117,118} single-strand conformation polymorphism (SSCP),^{155,173} or terminal restriction fragment length polymorphism (T-RFLP)^{93,130} of genes or gene fragments amplified from total community DNA. DGGE, SSCP, and T-RFLP are ideal for analysis of PCR products amplified from 16S or 18S rRNA genes composed of conserved and variable regions. PCR products of the same length but of different sequences can be separated by DGGE according to the melting behavior of the DNA or by means of SSCP due to their conformation polymorphism. For T-RFLP the 16S rDNA fragments are amplified with primers labeled with fluorochromes from total community DNA. The PCR products are subsequently digested with restriction enzymes. Due to the sequence differences in the variable regions, terminal restriction fragments of different lengths are obtained. Prominent bands can be excised from DGGE or SSCP gels, reamplified, and sequenced. The detection level of PCR-amplified ribosomal fragments may vary for several reasons, such as mismatches of the primer used for amplification with the respective target sequence or different numbers of rRNA operons. Often, more than one population might be hidden behind one band, which leads to an underestimation of the diversity.¹⁰⁹ Thus the number and intensity of bands (DGGE, SSCP) or peaks (T-RFLP) do not necessarily correspond to the cell number of a species. However, it should be kept in mind that molecular fingerprints of PCR-amplified 16S rDNA fragments have a lower resolution than sequencing of 16S rDNA clone libraries.¹⁰⁹ Using genetic fingerprinting techniques many studies have revealed shifts of bacterial communities during plant growth development, or shown plant-dependent diversities in the rhizosphere of different crop plants.^{40,41,87,151,156,161,195} Despite several pitfalls of PCR-based rRNA analysis, profiling of microbial communities by DGGE, SSCP, or T-RFLP have proven to be a rapid and relatively inexpensive method allowing a cultivation-independent analysis and comparison of large numbers of samples.

Cloning of 16S rDNA fragments amplified from community DNA by PCR combined with subsequent sequencing has opened a new era of cultivation-independent microbiology^{131,132} and has already been widely applied to study microbial diversity in soils. Although labor-intensive and time-consuming, the extended molecular approaches based on cloning and sequencing of 16S rDNA, fragments, which are PCR-amplified from community DNA have provided most valuable information on the large microbial diversity in soil. A substantial bacterial diversity was revealed with minor or no representation by cultured bacteria.⁶⁹ The analysis of 16S rDNA clone libraries from different soils revealed an enormous diversity and confirmed predictions based on DNA reassociation studies.¹⁷⁵ Usually, little redundancy among sequenced 16S rDNA from soil is found.¹⁰⁷ The presence of duplicate sequences in soil libraries might be an indication of reduced diversity.

Furthermore, cloning and sequencing of 16SrDNA from both whole-community samples and isolates are very useful to confirm or interpret the diversity patterns obtained by molecular fingerprinting as described above. Without a cloning protocol, the microbial diversity of soils can also be analyzed by restriction analysis of 16S rDNA fragments amplified from soil DNA; this approach was used to study key determinants that drive microbial community structure in soil.²⁰²

6.2.2.3.4 *Phylogenetic Oligonucleotide Arrays*

To analyze the structural diversity of soil bacterial communities, DNA extracted from soil can also be spotted on membranes and hybridized with phylogenetic probes. Quantitative dot-blot hybridization with phylogenetic probes has thus been used in several studies to analyze the structural composition of soils exposed to different agricultural management practices.²⁴ Nucleic acid microarrays, which represent a further development of conventional membrane-based technology, offer the enormous advantage of multiplex detection. Thousands of genes can be simultaneously assessed by using large sets of probes fixed on glass slides. So-called phylogenetic oligonucleotide arrays

(POAs), which are constructed with short synthetic oligonucleotides from rRNA genes, can be used as an *in vitro* hybridization approach for a structural analysis of bacterial communities.²⁰¹ The application of micro-array hybridization in soil diversity studies poses a number of methodological challenges. Hence, when environmental DNA is used without prior PCR amplification, sensitivity is most critical since the level of detection is 1,000- to 10,000-fold lower than with PCR amplification.¹⁹³ The sensitivity can be improved, however, by using *in vitro* transcribed RNA or PCR-amplified 16S rDNA. Another critical parameter is the specificity, which is largely determined by the nucleotide composition of mismatches and the mismatch composition.

6.2.3 ANALYSIS OF THE ACTIVE MICROBIAL COMMUNITY

Despite the exciting new insights into the diversity of the composition of soil microbial communities based on the analysis of environmental DNA pools, very little is known about the diversity of subpopulations actually contributing to soil microbial activity in a given space and time. Linking the information on the composition of total and active populations in soil remains a methodological challenge, but several novel approaches have appeared. The first two, presented briefly in the following, are making use of the incorporation of labeled compound into DNA during replication, and thus provide assays of growth-active subpopulations in environmental samples. The others are based on detection of mRNA or rRNA taken to represent gene expression and growth activity in active subpopulations.

6.2.3.1 Bromodeoxyuridine (BrdU) Method

Bromodeoxyuridine (BrdU) is a structural analogue of thymidine. The uptake of [³H] thymidine has long been used routinely for measuring *in situ* growth of bacteria in different environments. Recently, BrdU incubation was used to detect metabolically active bacteria in microbial communities from lake-water¹⁷⁸ and soil.^{17, 197} The protocol consists of three steps: (1) incubation of environmental bacteria (soil or microbial fraction), (2) extraction of DNA directly from the environmental sample or the microbial fraction, and (3) immunocapture of the DNA with incorporated BrdU using magnetic beads covered with anti-BrdU antibody. Limitations of the BrdU method might be a nonspecific binding to the magnetic beads of DNA that did not incorporate BrdU, or presence of bacteria unable to incorporate BrdU. While the majority of bacteria is supposed to take up and incorporate [³H] thymidine, this is not fully studied for its analogue BrdU. Artursson and Jansson⁶ used the BrdU method to identify actively growing bacteria from fallow soil containing arbuscular mycorrhizae. Using a combination of molecular and traditional approaches these authors were able to isolate, identify, label, and visualize a specific bacterium that was active in the soil, associated with arbuscular mycorrhizal spores.

6.2.3.2 Stable Isotope Probing

The stable isotope probing (SIP) is a promising strategy in particular to cultivation-independent identification of microorganisms responsible for certain biogeochemical transformation processes *in situ*.^{18, 50} This technique was first used to identify ¹³C-enriched PLFA signature profiles, for example, in experiments aiming to identify microbial populations responsible for acetate oxidation in sediments.¹⁹ A major advance in linking structural diversity and function was thus achieved with the nucleic acid-based SIP. Radajewski et al.¹⁴⁰ pioneered this approach and showed that ¹³C-DNA produced during growth of microbes on a ¹³C-enriched carbon source can be separated from ¹²C by density gradient centrifugation. Possible limitations of the method might be the dilution of the labeled substrate before its incorporation¹⁴⁰ and its dependence on the DNA synthesis rate *in situ*, which reflects the replication of bacterial cells. When the SIP approach was applied to study methanol utilizing microorganisms in soil, the involvement of α -proteobacteria and *Acidobacterium* lineages could be demonstrated. The analysis of heavy ¹³C-labeled DNA obtained after incubating

peat soil with $^{13}\text{CH}_4$ by PCR amplification, cloning and sequencing of 16S rRNA genes and the genes encoding the methane monooxygenase (pMMO, sMMO) and the methanol dehydrogenase (MDH) enabled the identification of functionally active methanotrophs.¹¹⁴ The analysis has recently been extended to 16S rRNA analysis or reverse transcription PCR in fractions of the density gradient followed by either denaturing gradient gel electrophoresis^{103,191} or construction of clone banks.¹⁰⁰¹⁰¹ This approach is dependent on a rather efficient accumulation activity and labeling efficiency of the active cells to be separated from the nonactive cells.

6.2.3.3 Reverse Transcriptase PCR (RT-PCR)

Detection of mRNA in environmental RNA by reverse transcriptase-PCR (RT-PCR) is potentially a powerful tool to assess gene expression and thus active subpopulations in soil, but several shortcomings might affect the results. Besides the well-known biases of PCR from environmental nucleic acids,¹⁸⁶ the most critical factor is the stability of the transcripts. Nogales et al.¹²⁴ assessed the bacterial diversity of PCB-polluted soils from clone libraries generated from both rDNA and rRNA. A good correlation of the community composition in both types of libraries and a high bacterial diversity was found even in PCB-contaminated soil. Several other studies have already utilized the RT-PCR to investigate expression of different functional genes.⁹² The RT-PCR-based approach was used successfully to study both the diversity and expression of five key enzymes involved in bacterial denitrification in river sediments.¹²⁵ Although this approach can currently be successfully applied, it cannot be regarded as routine assay and seems to be limited to transcripts expressed at a high level.¹²⁵ However, after proper adjustment of the nucleic acid extraction method of Griffiths et al.,⁵⁴ mRNA can be retrieved from soils and converted to cDNA for further molecular diversity analysis.^{5, 159}

6.2.3.4 Dot-Blot Hybridization

The relative abundance of active taxa can also be determined by hybridization of dot-blotted RNA with a specific probe and a universal probe, followed by calculation of the ratio of their respective hybridization signals. Here, both coextracted humic substances and DNA affect RNA hybridization results with oligonucleotide probes. Furthermore, different regions of the ribosomal RNA have different susceptibility to attack by RNases, which might result in a partial loss of probe or primer target sites. Such a partial RNA degradation is most critical when RNA is used for quantitative hybridization.¹

6.2.3.5 Fluorescence *In Situ* Hybridization (FISH)

Finally, an alternative measure of active populations in environmental samples is available by the much-applied fluorescence *in situ* hybridization (FISH) technique, in which the ribosomal RNA of intact cells within the samples is targeted directly using fluorescence-labeled phylogenetic oligonucleotide probes. The high number of ribosomes (and thus rRNA and high fluorescence intensity in FISH) is taken to indicate growth-active cells in the sample.³ Over recent years, more studies have used a combination of several fluorescent phylogenetic probes at different levels of specificity (e.g., group, genus, and species levels) simultaneously to improve the reliability of the identification in complex samples. However, the sensitivity of the standard FISH protocol is certainly a major limitation, but it can be improved by several recent developments,¹⁸⁸ as summarized below. Since the accessibility of particular target sites vary strongly within the 16S rRNA molecule and in different bacteria, the optimization of the probes should be performed using, for example, the tool available at the Probe Base website⁹⁶ to get the best performance possible. Also the use of so-called “helper probes” should be regarded for certain target sites at the 16S rRNA to increase the fluorescence intensity considerably.⁴⁴ Alternatively to rather short oligonucleotide probes multilabeled poly(ribo)nucleotide probes for FISH gave a very significant improvement of

the cell labeling,²⁰³ allowing for detection of even single-gene copies in single cells.²⁰⁴ Another very useful tool is the enzymatic signal amplification cascade using polyribonucleotide probes and the catalyzed reporter deposition (CARD-FISH) technique.^{153,135} The application of CARD-FISH for microbial communities in complex structured soil microhabitats has to be optimized individually. With the combination of CARD-FISH-analysis with impregnation and embedding of the soil samples with polyester resin, bacteria could be identified in their authentic soil microenvironment in thin sections microscopically.⁴² The combination of FISH-analysis with the application of radiolabeled substrates enabled the concomitant detection of the specific uptake and utilization of these substrates and identification of populations involved in this *in situ* activity using microautoradiography and CLSM analysis.⁹⁰

6.2.3.6 Confocal Laser Scanning Microscopy (CLSM)

Much of the recent advances in rhizosphere microbiology has been obtained by fluorescence microscopic recordings of cellular identity, localization, and metabolic activity in the same specimen, all observed at a single-cell level. In environmental samples, image analysis supported microscopy like confocal laser scanning microscopy (CLSM) or the deconvolution microscopy are necessary to reduce autofluorescence or the fluorescence from out-of-focus planes.¹⁵² This optical sectioning of the specimen allows projections of a series of xy-scans and results in z-projections, which can be analyzed as a 3D-image, for example, in the orthogonal viewing mode. Using computer-controlled motorized stage the observation fields on the objective slide can be located precisely and reproducibly; thus microscopic observation and image acquisition can be randomly selected.⁸⁵ Spiking the samples with known amounts of bacterial cells was successfully used to determine the absolute number of cells in a complex sample.^{30, 31} Finally, the detailed analysis of bacterial communities documented by CLSM images can be analyzed for bacterial morphotypes and spatial arrangements using the CMEIAS computer software.^{32,94}

The first applications of CLSM to study bacterial root colonization were based on strain-specific fluorescent antibody staining to follow the inoculants.^{33,56, 148} Counterstaining of total bacterial populations using DAPI was also included at an early stage.¹⁴⁹ The first combined application of FISH and CLSM in the rhizosphere identified and localized an *A. brasilense* inoculant strain in the rhizosphere of wheat grown in soil.⁷ This study showed a high ribosome content of pleiomorphic *Azospirillum* aggregates on the rhizoplane and some root hairs completely filled with the inoculant. In the same year FISH and CLSM was also applied in a sludge system.¹⁸⁷ Co-inoculation experiments demonstrated that *Azospirillum brasilense* strains colonizing wheat roots were mutually competitive,⁹ while *Pseudomonas fluorescens* strains colonizing barley roots were not.⁵⁶ Assmus et al.⁹ used combinations of strain-specific monoclonal antibody (*Azospirillum brasilense* Wa3), species-specific FISH probe (*A. brasilense*), group-specific FISH probe (alpha-class of Proteobacteria), and general FISH probe (domain Bacteria) on wheat roots and demonstrated the potential of combining probes of different specificity. A second example using group-specific FISH probes is provided by the studies of Upton et al.¹⁷⁷ and Gilbert et al.⁴⁹ addressing methanogenic and methanotrophic bacteria, respectively, in waterlogged soil environments. Using a FISH probe targeting the whole domain Bacteria (e.g., probe EUB338), total populations of active bacteria have been enumerated in both soil samples²⁸ and in the rhizoplane of young seedling roots⁹⁹; the latter work described the early colonization events on sugar beet roots for both a seed-inoculated *Pseudomonas fluorescens* strain and the native soil bacteria. Schallmach et al.¹⁴⁷ used a group-specific FISH probe (rRNA group I pseudomonads) to follow the effect of nitrogen deficiency on colonization of common bean roots. Apart from FISH- or immunolabeling, fluorescent marker or reporter genes like *gfp* (green fluorescent protein) or *rfp* (red fluorescent protein) are now frequently used as illuminating tools for *in situ* analysis of specific populations or microsite conditions.^{27, 76, 91,170} The specific diversity of nitrogen-fixing bacteria and Pseudomonads in the rhizosphere addressed in the following paragraph.

6.3 PLANT-BENEFICIAL RHIZOSPHERE BACTERIA

The knowledge about the importance of root-associated bacteria for plant growth started with the discovery and proof of symbiotic nitrogen fixation of soil bacteria with legume plants already in 1886 by Hellriegel and Wilfarth. Shortly after, this knowledge about plant-beneficial bacteria was taken into biotechnological application by the development of specific inoculants (“nitragin”) for different legumes in the 1890s and the early 20th century by Hiltner.⁶⁵ The breakthrough was the discovery of the specificity of the rhizobia–legume combinations, making the inocula extremely effective. At that time, the importance of pathogen control in the rhizosphere and of suppressiveness in certain soils were already observed as were the potential of plant growth promotion in nonlegumes.⁶⁷ In the following decades continuous progress was made in revealing the special nature of the rhizosphere (e.g., using improved microscopic methods).²⁰ However, it was not before the advent of molecular biological tools in microbial ecology that more details of the microbial population structure of the rhizosphere and different mechanisms of plant beneficial effects of rhizosphere microbes were discovered.

In the next section, we address specific groups of rhizosphere bacteria, supporting either plant nutrition and growth by nitrogen fixation and/or improved nutrient uptake, or plant protection against pathogens by induction of improved resistance and/or antimicrobial activity (biological control). The diversification of the microorganisms, both in relation to their natural function and to their prospect as novel plant-beneficial organisms, is discussed.

6.3.1 SYMBIOTIC NITROGEN FIXING BACTERIA IN AGRICULTURE

Although inorganic fertilizer appears quite available, inexpensive and easy to use for modern agriculture, the enhanced involvement of BNF in modern cropping systems has importance for sustainability of farming systems. Ecological and organic agriculture is growing constantly in industrialized countries. This aim for more holistic food production, where external synthetic crop production inputs are reduced, presents an important opportunity to expand the role of BNF in modern agriculture representing a low-input approach. BNF from legumes increases the biological efficiency of crop production through direct supply of N to the soil system and through many indirect benefits of improved soil fertility associated with perennial cropping and pasturing. In many grain-exporting countries such as the United States, Canada, Australia, Argentina, and others, grain production has relied to a significant effect on indigenous soil N supply, called “N-mining”-effect.⁴³ Therefore, there is a need to fill up the N stores of these N-deficient soils, at best by incorporation of leguminous plants in the crop rotation or by intercropping. For the future of BNF from legumes it has been predicted that the contribution will continuously rise (e.g., in Western Canada) to 550 million kg of N annually by 2005, which is almost one-third of the total inorganic N fertilizer used in Canada in 1996.⁴³

6.3.1.1 Rhizobium–Legume Symbioses

The main legumes used in agriculture are listed in table 6.1.

The production of grain legumes (peas and lupines) has increased dramatically worldwide in the past 20 years. While only modest gains in soybean and dry pea production were recorded for EU countries, major soybean producers are the United States (29 million ha) and Brazil (12.9 million ha). Most grain legumes derive a majority of their nitrogen from nitrogen fixation and it is estimated that legumes provide 25–35% of the worldwide protein intake.⁴³ By comparison, forage legumes are often grown in combination with perennial forage grasses (e.g., *Festuca* spp. and *Lolium* spp.) and are rotated with cereal and oilseed grain crops often in a 6- to 8-year rotation. Fallow periods are often included in grain production; green fallow refers to a practice where short-duration legume crops are grown during fallow periods. In tropical countries, *Sesbania rostrata* is planted as green manure to enrich soils with nitrogen. In this way, BNF by legumes can contribute a N-fertilizer replacement of up to 150 kg ha⁻¹.

TABLE 6.1
Leguminous Plants for Biological Nitrogen Fixation (examples)

Plant host	Microbial symbiont
Grain legumes	
Soybean (<i>Glycine max</i> L.)	<i>Bradyrhizobium elkanii</i> , <i>Sinorhizobium fredii</i>
Pea (<i>Pisum sativum</i> L.)	<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>
Lupin (<i>Lupinus</i> spp.)	<i>Rhizobium leguminosarum</i> bv. <i>lupini</i>
Bean (<i>Phaseolus</i> spp.)	<i>Rhizobium tropici</i> , <i>R. etli</i>
Common Bean (<i>Phaseolus vulgaris</i>)	<i>Rhizobium tropici</i>
<i>Vicia sativa</i>	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>
Forage and pasture legumes	
Alfalfa (<i>Medicago sativa</i> L.)	<i>Sinorhizobium meliloti</i>
Red clover (<i>Trifolium</i> spp.)	<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>
Lotus (<i>Lotus</i> spp.)	<i>Mesorhizobium loti</i>
Green fallow, cover crop, and green manure legumes	
Chickling vetch (<i>Lathyrus sativus</i> L.)	<i>Bradyrhizobium</i> sp.
<i>Sesbania rostrata</i>	<i>Azorhizobium caulinodans</i> .

6.3.1.2 Diversity of Rhizobia

Root- and stem-nodule bacteria forming symbioses with legumes fall into four deep phylogenetic branches within the alpha-Proteobacteria: *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and the *Rhizobium-Sinorhizobium-Allorhizobium* group. The evidence for these four branches, including 6 genera and 27 species, is based on 16S- and 23S rDNA analyses and phenotypic features. Interestingly, nonsymbiotic relatives are closely phylogenetically related and situated within these four branches, pointing to possible common ancestors between symbiotic, parasitic, or saprophytic soil bacteria. At present, complete genome sequences are available from three species (*Bradyrhizobium japonicum*, *Mesorhizobium loti*, and *Sinorhizobium meliloti*; see also <http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>), providing the basis for an even more profound understanding of the relationships between different N₂-fixing symbionts. Apart from the analysis of the complete 16S rDNA sequences, the restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rDNA has been useful for estimating the phylogenetic position and for grouping a large number of isolates.¹⁹⁸

The genus *Azorhizobium* consists of only one species, *A. caulinodans*, which represents the bacteria nodulating on stems and roots of *Sesbania rostrata* in Africa³⁹; it is able to fix nitrogen also in the free-living state. *Xanthobacter* species, which are also plant-associated bacteria, some of which are able to fix nitrogen, are closely related to *Azorhizobium*. The genus *Bradyrhizobium* includes the slow-growing, alkali-producing species *B. japonicum*,⁷⁸ *B. elkanii*,⁸⁶ and *B. liaoningense*.¹⁹⁴ This group also includes many other species, including some photosynthetic, stem-nodulating, and pathogenic species.¹⁶³ *Bradyrhizobium japonicum* is closely linked to *Afipia* spp., *Blastobacter denitrificans*, and *Rhodopseudomonas palustris*. Interestingly, methanol-oxidizing bacteria *Methylobacterium* spp., some of which form N₂-fixing nodules with tropical legumes, also belongs to this group. The third branch of rhizobia harbors the Mesorhizobia, including seven described species of slow- or moderately slow-growing, acid-producing bacteria, such as *M. ciceri*, *M. huakuii*, *M. mediterraneum*, and *M. tianshanense*. The former species *Rhizobium loti* has become the type species of this group as *Mesorhizobium loti*.⁷⁷ These bacteria form a group together with methylotrophic species of the genera *Aminobacter* and *Chelatobacter*, inhabiting the plant rhizospheres as well as *Phyllobacterium*. The genera *Ochrobactrum* and *Brucella*, known as opportunistic human pathogenic bacteria, are related more distantly to the Mesorhizobia branch. However, many soil and rhizosphere associated isolates from the *Ochrobactrum* species *O. anthropi*, *O. grignonense*,

and *O. triticum* are now known.⁸⁸ Some isolates were recently demonstrated to be able to form effective nodules¹²² or to live endophytically in roots of rice.¹⁸³

The remaining branch includes the three symbiotic genera *Allorhizobium*,³⁴ *Rhizobium*,¹⁹⁴ and *Sinorhizobium* and the plant pathogenic *Agrobacterium* species, *A. radiobacter* (or *A. tumefaciens*), *A. rubi*, and *A. vitis*.¹⁸⁹ The six species of the genus *Sinorhizobium* form a narrow cluster sharing a common ancestor with species of *Allorhizobium* and *Rhizobium*. These genera are closely related both in genetic and phenotypic aspects.¹⁹⁸ The phylogenetic relationship among rhizobia has also been investigated using 23S rRNA sequences¹⁷⁴; this approach mostly confirmed the 16S rDNA-based phylogenetic tree. Only the relationship of *R. leguminosarum* with *Agrobacterium vitis* appeared not as closely based on 16S rRNA sequence comparison.

6.3.1.3 Host Specificity in Rhizobia–Legume Symbioses

There is a high specificity of the so-called fast-growing *Rhizobium* species or biovars for the symbiotic interaction with certain leguminous plants (table 6.1). Therefore, inoculations with commercial *Rhizobium* inoculants are recommended. The so-called slow-growing *Bradyrhizobium* spp. has a more broad symbiotic host range from soybean to the nonleguminous *Parasponia* tree.

The host specificity of rhizobia is determined mostly by the *nod* genes. These symbiotic genes are located on potentially transferable Sym plasmids in all species of *Rhizobium* and *Sinorhizobium*, as well as in *Mesorhizobium amorphae* and *M. huakuii*. In *M. loti* and *B. japonicum*, they are placed on transferable chromosomal elements. The symbiotic genes have clearly different evolutionary histories as compared to 16S rDNA genes. The relationship between the host range of rhizobia is reflected in differences of the *nodA* and *nodC* gene sequences as was found, for example, within the genus *Sinorhizobium*.^{61,176} Two groups of *nod* genes were found in *Bradyrhizobium* and *Rhizobium*³⁶; one determined the symbiosis with temperate legumes of the tribes *Viciae* and *Trifoliae*, the other with tropical legumes in the tribe *Phaseolae*. This clearly indicated close relationships between the common *nod* genes and the host ranges of the bacteria. The recognition of the rhizobial Nod-factors by the host plant could also result in a similarly directed legume phylogeny.

6.3.1.4 Competitive Inoculant Strains

Due to indigenous rhizobial strains, which may be better competitors for infection and subsequently for nodule occupancy,¹⁴⁴ highly competitive inoculant strains with high nitrogen fixation potential are often necessary for successful use of BNF in legume crops. Hence, indigenous strains often exist naturally in soil at high population levels and with a wide diversity of rhizobial strains. This diversity allows a rhizobial subpopulation to be available for nodulation at a wide range of environmental conditions, while the inoculum strains may be best suited for efficient nodulation under a limited temperature or moisture range, thus reducing the likelihood for symbiosis.¹⁸⁴ Thus, competitiveness of inoculants as well as the saprophytic survival of strains under stress situations should be as high as possible. One approach is the use of bacteriocin-producing Rhizobia. Bacteriocins are small peptides that inhibit growth of closely related bacteria specifically. Trifolitoxin is the best-understood bacteriocin involved in the competition of rhizobial strains and it could be demonstrated that it indeed increases nodulation of *Rhizobium etli* in *Phaseolus vulgaris*.¹⁴³ Since trifolitoxin does not inhibit bradyrhizobia, this approach cannot be generally applied.

Nodulation competition in the rhizosphere may further be improved by the production of unique carbon sources either by the plant host or the symbiont. The competitive advantage is conferred on *Rhizobium* strains able to use the carbon source over strains unable to use it. Rhizopines are inositol-like compounds made by *Sinorhizobium meliloti* and *Rhizobium leguminosarum*.¹¹⁶ The genes of synthesis and catabolism of rhizopines are located on the Sym plasmid. It could be demonstrated that the ability of rhizopine utilization resulted in a significantly higher

nodule occupancy and strains able to catabolize rhizopines remain dominant in alfalfa nodules over years.

Finally, another possibility of increasing legume productivity is possible through the presence of the hydrogen uptake phenotype, because the escape of hydrogen, which is an unavoidable byproduct of the nitrogenase reaction from nodules, can be minimized.¹⁰² On the other hand, it could be demonstrated by Dong and Layzell³⁸ that the hydrogen released by the nodule increases the microbial activity in the legume rhizosphere, which causes a general plant growth stimulatory effect.^{37, 110} In addition, the rhizosphere bacteria start to fix carbon dioxide under the influence of this hydrogen gas, which could be regarded as another beneficial trait of a legume crop. Concomitant with this hydrogen-induced stimulation of the microbial activity and biomass, population shifts were observed using group-specific phylogenetic probes and FISH-analysis.¹⁶⁷

6.3.1.5 Unrevealed Diversity of Rhizobia and Novel Symbiotic Nitrogen-Fixing Symbioses

Based on the evidences for horizontal transfer of the symbiotic genes, it is quite probable that some rhizobia evolved from nonsymbiotic ancestors. The discovery of *Lotus*-nodulating bacteria, which have undoubtedly evolved from indigenous non-nodulating *Rhizobia* by acquiring the symbiotic genes from an inoculant strain via chromosomal gene transfer,¹⁷¹ suggests that new root-nodulating bacteria are still emerging in nature. A similar situation may be present in *Agrobacterium* species, where the presence of T_i (tumor inducing) or R_i (hairy-root inducing) plasmids determine the plant pathogenic phenotype, because nonpathogenic strains have been found within all *Agrobacterium* spp.¹⁹⁸

The transfer of symbiotic genes also reached bacteria outside the Rhizobiaceae and the alpha-Proteobacteria. The beta-Proteobacteria *Burkholderia tuberum* and *B. phymatum*¹⁸⁰ were demonstrated to be nodule-forming bacteria in certain tropical legumes.¹¹⁵ It has been demonstrated that they harbor *nod* genes closely related to the *nodA* genes of Rhizobia. Once the *nod* genes have been acquired by a rhizosphere bacterium capable of interacting tightly with a legume plant host via horizontal gene transfer, their further evolution could have been under functional constraints of the plant.

High rates of BNF have also been reported in some gramineous plants, which—according to textbooks—do not support effective nitrogen fixation like the Rhizobia and their symbiosis in legumes. For example, highly efficient N_2 -fixing sugar cane varieties have been described, which are able to derive 60–80% of their nitrogen from fixation of atmospheric nitrogen.¹⁵ Diazotrophic bacteria of different genera (*Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia*) were found to colonize endophytically and systemically these plants.^{58, 158} They are providing the plant with fixed nitrogen derived from atmospheric N_2 with different levels of efficiency.¹⁴ The exact mechanism of this new type of nitrogen-fixing symbiosis has not yet been identified unequivocally.⁷¹

6.3.2 PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

A plant growth stimulatory effect due to inoculation with rhizobacteria has been observed for several decades. Plant growth can be promoted by direct and indirect mechanisms. Hence, while the latter is due to, for example, plant-protecting microorganisms (further treated in a separate section below), the direct effect is due to production by certain bacteria of specific growth-stimulating compounds such as phytohormones or interaction of rhizosphere microbes with the ethylene metabolism of the plant.⁵¹ It has been estimated that up to 80% of all rhizosphere bacteria are able to produce the phytohormone indole acetic acid (IAA, “auxin”).⁵ A large number of rhizosphere-inhabiting bacteria may thus potentially produce phytohormones. For the present purpose we will focus on nitrogen-fixing bacteria, like *Azospirillum* and *Azoarcus*, which have been studied intensively. The other genus emphasized here is the *Pseudomonas*, which in turn also support plant growth and health by their role in plant protection against pathogens.

6.3.2.1 Nitrogen-Fixing PGPR

One intensively studied root-associated diazotrophic bacterium is *Azospirillum*. Five species are known: *A. brasilense*, *A. lipoferum*, *A. doebereineriae*, *A. amazonense*, and *A. halopraeferens*.^{82,168} Some *Azospirillum* strains are able to colonize not only the rhizoplane but can enter the rhizodermis and colonize the apoplast or occasionally rhizodermal cells and root hairs of, for example, wheat plants. This has been demonstrated using strain-specific antibodies,¹⁴⁹ FISH,⁷ and gfp¹⁴⁶-labeling studies. *Azospirillum* produces not only auxins such as IAA and related compounds but also in smaller amounts gibberellic acids (GA₃) and cytokinins.⁴⁵ When colonizing the root surface, the bacteria produce these compounds and as a consequence, roots of, for example, inoculated maize seedlings, have higher amounts of both free and bound IAA and indole-3 butyric acid (IBA) than control plants. This increase in free IAA occurs in roots of inoculated seedlings already two weeks after sowing. Likewise, GA₃ was found in free acid form in roots of seedlings inoculated with *Azospirillum lipoferum*. The inoculation effect using an optimal number of cells in a common peat-based inoculum, like in the commercially available product Azogreen[®], results in a general improvement of root and early shoot development. In the case of lighter soils with low organic matter contents and only low to intermediate levels of fertilizers (N, P, K) and limiting water regimes, *Azospirillum* inoculation resulted in 5–30% yield increases in maize, wheat, and sorghum in several parts of the world.³⁵ In heavier soils and under high levels of fertilizer, the early growth stimulation effect is usually not translated into yield. However, an improved N-uptake from the soil could be demonstrated in maize fields in Europe after application of Azogreen[®]. In *Rhizobium*-inoculated legumes, commercial *Azospirillum* co-inocula resulted in a 15–30% yield increase above legumes inoculated with *Rhizobium* alone.³⁵ The stimulation of nodulation following *Azospirillum* inoculation could be due to the observed increase of lateral roots, root hair density, and branching and to the differentiation of more epidermal cells into root hairs susceptible for infection by rhizobia. *Azospirillum* was also found to cause an increased secretion of root flavonoid substances, known to be involved in the activation of nodulation.¹⁸⁵

A wide diversity of endophytic bacteria is known in all kinds of plants, exerting nitrogen fixation, plant growth promotion, biological control, or even biodegradative potential for xenobiotics.⁹⁵ Some of the bacterial endophytes colonizing the root interior of crop plants of agronomical importance are thus able to fix N, for example, *Azoarcus* spp., several *Burkholderia* spp., *Gluconacetobacter* spp., and *Herbaspirillum* spp. (table 6.2).

Root endophytic bacteria probably enter the roots via lesions occurring where side roots penetrate the outer root layers; however, they may also penetrate through the intercellular spaces in the apoplast like in the case of *Azoarcus*.⁷⁰ The colonization density reaches 10⁶ per gram fresh weight in roots and 10⁵ to 10⁴ per gram fresh weight in stems and leaves, respectively. It has been shown with FISH methodology that endophytic bacteria are actively metabolizing, like in *A. brasilense*.¹⁴⁵ In the case of *Azoarcus* it has been demonstrated that endophytic bacteria express nitrogen fixation (*nifH*) genes *in planta*.¹⁴² For *Azoarcus* sp. BH72 and *Gluconacetobacter diazotrophicus* PAL3 there is even clear evidence that they substantially contribute fixed nitrogen to the plant.^{71,157} It has been shown with ¹⁵N dilution techniques that in certain rice varieties (like IR42 and IR28), up to 30% of the plant nitrogen is derived from atmospheric N₂. In certain sugar cane varieties, 60–70% of the biological fixed nitrogen is derived from the nitrogen fixation of endophytic diazotrophs.¹⁵ Detailed mechanisms of this new type of nitrogen-fixing symbiosis are not known yet. These nitrogen-fixing PGPR usually also exert a general growth-stimulating effect, usually by producing phytohormones.

6.3.2.2 Non-Nitrogen-Fixing PGPR

The colonization of the rhizosphere by the PGPR *Pseudomonas fluorescens* was studied in detail using gfp-labeled strains.¹²⁶ Phytohormone production is found in many rhizobacteria such as in

TABLE 6.2
Root-Associated Plant Growth-Promoting Diazotrophic Bacteria (examples)

Bacteria	Plant host
Rhizoplane and facultative endophytic diazotrophs in nonleguminous plants	
<i>Azospirillum brasilense</i>	Wheat, rice, forage grasses (<i>Panicum maximum</i> , <i>Digitaria</i> spp., <i>Brachiaria</i> spp.), sugar cane, palms
<i>Azospirillum lipoferum</i>	Cereals, forage grasses, sugar cane, palms, tubers
<i>Azospirillum amazonense</i>	Rice, wheat, sugar cane, palms
<i>Azospirillum irakense</i>	Rice
<i>Burkholderia vietnamiensis</i>	Rice
<i>Klebsiella</i> sp.	Maize
<i>Pseudomonas stutzeri</i>	Rice
Obligate endophytic diazotrophs in nonlegumes	
<i>Azoarcus</i> spp.	Kallar grass, rice
<i>Burkholderia tropica</i>	Sugar cane
<i>Burkholderia brasiliensis</i>	Rice, cassava, banana, sweet potato
<i>Gluconacetobacter</i> spp.	Sugar cane, coffee
<i>Herbaspirillum</i> spp.	Cereals, rice, sugar cane, forage grasses, palms, C4-energy crops (<i>Pennisetum</i> spp., <i>Miscanthus</i> spp.)

Pseudomonas, *Serratia*, or *Paenibacillus polymyxa*.⁸⁹ A number of reports have documented a stimulating effect of IAA-producing *Pseudomonas* spp. on root development and plant growth. Beyeler et al.¹³ demonstrated that *P. fluorescens* CHA0 mutants (1,000-fold overproducing IAA) inoculated with cucumber plants in natural soil gave almost 20% increase of fresh-weight root biomass. A clear result of this type of plant–microbe interaction is the elevated concentration level of IAA and of IAA-producing bacteria in the rhizosphere.⁵ It has long been asked if the *Pseudomonads* produce phytohormones to stimulate plant growth and thus exudation of easily-accessible C substrate for their own growth. Another advantage could be the increased root development, simply providing more space for colonization and thus excess to root exudates. Finally, to understand the regulation of bacterial phytohormone production in the rhizosphere, it is important to identify any plant-derived molecular signals, which induce bacterial hormone production. Benizri et al.¹¹ showed that root exudate from corn (*Zea mays*) stimulated production of IAA in a *P. fluorescens* strain under *in vitro* conditions. At present, there is much evidence that an optimal IAA concentration in the rhizosphere is critical to growth stimulation, and much has yet to be learned on how such a balanced hormone level is reached in the rhizosphere, for example, during application of microbial inoculants.

Certain *Pseudomonas* spp. further produce the enzyme ACC-deaminase, hydrolyzing the ethylene precursor ACC. This activity may reduce the root growth-inhibiting ethylene concentration in the rhizosphere, thus causing a plant growth-promoting effect.¹³³ Finally, an interesting aspect of regulatory mechanisms is the fact that IAA stimulates production of the ethylene precursor ACC; on this background it is understandable, that regulation mechanisms for the plant–microbe interaction via the phytohormone IAA and the ethylene precursor ACC are indeed complicated.¹³⁴ When clearly understood, however, they may provide a useful asset for application of *Pseudomonas* spp. as PGPR inoculants. Thus, it could be demonstrated that PGPR could successfully be used to confer resistance to water stress in tomatoes and peppers.¹⁰⁷

Since the *Pseudomonas* spp. are similarly important in plant protection (as described in the next paragraph), a treatise of their diversity in soil and rhizosphere will be made later.

6.3.3 PLANT-PROTECTING RHIZOBACTERIA

Several strategies of plant protection by microorganisms have been identified and these are practically used by treatments increasing the natural resistance of the plant or providing an elevated population of biocontrol-active microorganisms (table 6.3).

Whether the strategy is based on stimulation of the indigenous microflora or on use of microbial inocula, there is a great demand for mechanistic understanding of the plant–microbe interaction and for optimal selection of efficient strains or combinations of strains for plant protection. In the following, we will treat the microorganisms providing a higher plant resistance to pathogens separately from those performing a direct antagonistic action (biocontrol). Special emphasis will again be given to the *Pseudomonas* spp. group of rhizosphere bacteria, often possessing an astonishing variety of molecular mechanisms for plant protection.

6.3.3.1 Induction of Plant Systemic Resistance

Pathogenic rhizobacteria and related avirulent strains can induce systemic resistance in plants, which is termed “systemic acquired resistance” (SAR). All plants possess active defense mechanisms against pathogen attack,⁷³ both on a local and systemic level. The plant recognizes potential pathogens through elicitors such as certain surface structures like lipopolysaccharides or diffusible compounds. If the defense mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease can be reduced. This resistance occurs naturally as a result of a limited infection by a pathogen, when the plant develops a so-called hypersensitive reaction, which is usually a local response, also called locally acquired resistance (LAR). Characteristic for the systemic response is the production and accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs).¹⁸¹ Exogenous application of SA also induces SAR in several plants. Some of the PR-proteins are β -1,3-glucanases and chitinases and capable of hydrolyzing fungal cell walls.

Nonpathogenic rhizobacteria can further induce a systemic resistance in plants that is mechanistically different from the SAR-response¹³⁷ (table 6.3). This induced systemic resistance (ISR)

TABLE 6.3
Mechanisms of Microbial Control of Plant Pathogens (with some examples)

Mechanism	Biocontrol agent	Pathogen
Stimulation of plant systemic resistance		
Induced systemic resistance (ISR)	<i>Pseudomonas fluorescens</i> <i>Pseudomonas putida</i> <i>Serratia marcescens</i>	<i>Pseudomonas syringae</i> <i>Fusarium oxysporum</i> <i>Colletotricum orbiculare</i>
Systemic acquired resistance (SAR)	<i>Pseudomonas syringae</i> (avirulent)	<i>Pseudomonas syringae</i>
Nutrient competition		
Siderophores	<i>Pseudomonas putida</i> <i>Pseudomonas aeruginosa</i> <i>Serratia</i> spp.	<i>Fusarium oxysporum</i> <i>Pythium splendens</i> <i>Verticillium dahliae</i> , <i>Sclerotinia sclerotiorum</i>
C-substrates	<i>Agrobacterium radiobacter</i>	<i>Agrobacterium tumefaciens</i>
Antibiosis		
antibiotics	<i>Pseudomonas fluorescens</i> <i>Pseudomonas putida</i>	<i>G. graminis</i> var. <i>tritici</i> <i>Rhizoctonia solani</i>
HCN	<i>Pseudomonas fluorescens</i>	<i>G. graminis</i> var. <i>tritici</i>
Parasitism/predation		
chitinase	<i>Serratia plymuthica</i>	<i>Verticillium dahliae</i>
hyperparasitism	<i>Trichoderma harzianum</i>	<i>Pythium</i> , <i>Phytophthora</i>

can be effective against both fungi and bacteria (and viruses) in, for example, *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato, under conditions where the inducing bacteria and the challenging pathogen remain spatially separated. So far, induced systemic resistance has not been demonstrated in monocotyledons. Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). In the long-distance signaling of the ISR response, ethylene and jasmonic acid is involved.¹³⁷ Most recently it became apparent that N-Acyl-homoserine lactones (AHL), which are essential signal molecules in the “quorum sensing” regulated control in Gram-negative bacteria, can also trigger a systemic plant response similar to the ISR response.⁶⁰ AHL-producing *Serratia liquefaciens* MG1, as well as the signal molecule hexanoyl homoserine lactone itself, could trigger a systemic response in tomato plants. This induced systemic resistance was manifested in a reduced sensitivity of the tomato plants toward the attack of the foliar pathogenic fungus *Alternaria alternata*.¹⁵⁴ Examples for ISR-inducing bacteria are *Pseudomonas aeruginosa* 7NSK2, inducing resistance against *Botrytis cinera* (gray mold) or *Colletotrichum lindemuthianum* (anthracnose) in bean; *P. fluorescens* WCS472, protecting carnations against *Fusarium* wilt; or *Serratia marcescens* 90-166, protecting, for example, cucumber against cucumber mosaic virus, *Fusarium oxysporum* or *Erwinia tracheiphila*.¹⁸¹ SAR and ISR constitute a nonspecific protection against fungi, bacteria, viruses, and occasionally also against nematodes and insects. In addition, some of these bacteria were also found to produce phytohormones and may occur endophytically in the root cortex. Therefore, these biological control agents are also PGPR (plant growth promoting rhizobacteria), because the control of phytopathogenic bacteria results in increased plant growth even at pathogen levels that do not cause a phenotypically sick plant.

6.3.3.2 Biological Control of Plant Diseases

Microbiological control of plant diseases leads to the suppression of plant pathogens without the use of chemical plant protection. Survival and rhizosphere competence are major factors determining the longevity of biocontrol agents (BCA) at the places where they are most needed. In the rhizosphere, the source of nutrients to support BCA activity are root exudates or sloughed root cells. The very extensive literature on the fate and activity of microbial inoculants in the rhizosphere will not be reviewed here; reference is made to the latest studies that have elucidated the molecular mechanisms of biocontrol under natural soil conditions (role of plant and fungal signals for inoculant activity in root colonization, growth activity, metabolite production, etc.). Instead, a treatise is given here to the diversification of biocontrol organisms and to their occurrence in nature including disease-suppressive soils. The latter is important for recruitment of novel organisms for biocontrol.

6.3.3.2.1 Inoculants as Biocontrol Agents

A great diversity of soil microorganisms have been tested as biological control agents against soilborne or foliar plant pathogens. These biocontrol microbes are used in agrotechnological applications to limit pathogenic bacteria and fungi as well as plant-parasite nematodes.⁸² An increasing number of antagonistic bacteria are successfully applied in commercial biocontrol products, like Rhizostar[®], Phytovit[®], and Proradix[®]. On the other hand, many have only provided a limited effect or simply failed to survive long enough to have any significant effect. One possibility to improve efficacy of plant-protecting microorganisms is to introduce a food base with the microorganisms that supports their activity without stimulating the pathogen.⁶⁸ Here the concentration of available nutrients within the soil organic matter (carbohydrates, lignocellulosic substances, chitin, lipids, etc.) plays a critical role. As this organic matter decomposes, the microbes' carrying capacity of the amendment decline and the suppression is lost. Therefore, organic amendments such as green manure, stable manures, and composts can provide this food base and have long been recognized to improve the efficacy of antagonistic bacteria when applied well ahead of planting. In contrast, peat as the sole organic component in plant potting mixes does not support the suppression of plant pathogens, because it is low in readily available carbon and energy reserves for microbes. Such plant health

management practices can be very effective in controlling diseases caused by many soilborne plant pathogens ranging from *Pythium*, *Phytophthora*, *Fusarium* spp. to *Rhizoctonia solani*.

One of the mechanisms outlined for bacterial control of plant pathogens is the competition during colonization at the plant surface (table 6.3); the control agent may here utilize the growth substrate more efficiently than the pathogen. A specific case in this respect is the complexation of ferric iron by so-called siderophores, which are organic molecules of different structure produced by many microbes. The microbes producing the most efficient siderophores take away this essential micronutrient from competing populations. Therefore, the most active biocontrol bacteria may produce highly efficient siderophores; many fluorescent *Pseudomonas* strains thus excrete the catechol-based siderophores pyochelin or pseudobactin.⁹⁸ This leads to effective biological control of, for example, *Fusarium oxysporum* by *Pseudomonas putida*. In *Pseudomonas aeruginosa* pyochelin contributes to the biological control of *Pythium splendens* in tomato plants. In *Serratia* spp. several siderophores are produced under iron limitations such as the catechol enterobactin, the hydroxamate aerobactin, and ethylene diamine di-O-hydroxyphenylacetic acid; *Serratia* spp. are antagonistic against the *Verticillium dahliae*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*.

Many biocontrol microbes produce compounds with bactericidal or fungicidal compounds. Antibiotics like phenazine, pyoluteorin, 2,4-diacetyl phloroglucinol (DAPG), or pyrrolnitrin are produced by *Pseudomonas*, *Burkholderia*, *Enterobacter*, or *Pantoea*. In *Pseudomonas fluorescens* CHAO, bacterial production of HCN has additionally been identified as part of the biological control mechanism. In other *P. fluorescens* or *P. putida*, production of biosurfactant compounds (cyclic lipopeptides) has recently been assigned an important role in biocontrol of plant-pathogenic fungi in the rhizosphere.¹²⁸ Yet another mechanism behind biological control is the production of extracellular enzymes, such as β -1,3- glucanases, proteases, or chitinases. This was found in some antagonistic bacteria such as in *Enterobacter agglomerans*, which are able to attack fungal cell walls.²⁶

6.3.3.2.2 Indigenous Microorganisms and Suppressive Soils

Indigenous soil microorganisms may also contribute to the suppression of pathogens through all three mechanisms described above: (1) competition, (2) systemic-induced resistance, and (3) antibiosis. It has long been known that certain soils are naturally able to suppress the development of soilborne plant pathogenic fungi such as *Fusarium*, *Gaeumannomyces*, *Pythium*, *Phytophthora*, or *Verticillium*. In these soils different populations of competitors are present that inhibit the growth of the pathogens by several mechanisms. From the rhizosphere of wheat suppressive to *Gaeumannomyces graminis* var. *tritici*, causing the take-all disease, DAPG-producing *Pseudomonas fluorescens* isolates made up to 12% of the *Pseudomonas fluorescens* isolates and superior root-colonizing strains could be identified.¹⁴¹ The abundance and composition of antagonists of, for example, *Verticillium* in the rhizosphere, is plant species-dependent.¹² While a rather high proportion of antagonists from the strawberry rhizosphere belong to *Pseudomonas putida*, antagonists from the rhizosphere of oilseed rape belong to the *Enterobacteriaceae* (*Serratia* spp., *Pantoea agglomerans*).

6.3.3.3 Diversity of Novel Plant-Protecting Bacteria

Since *Pseudomonas* spp. are commonly exploited as beneficial bacteria leading to plant protection by induced systemic resistance or biological control as described above, we will shortly address their diversification in soil. Sørensen et al.¹⁶⁴ summarized a number of approaches based on modern technologies to study indigenous or inoculated *Pseudomonas* spp. populations in bulk soil and rhizosphere, for example, fluorescence stainings by cell surface-targeting antibodies or RNA-targeting oligonucleotide probes in combination with confocal laser scanning microscopy (CLSM), and gave specific examples on the use of inoculated *Pseudomonas* spp. strains (equipped with reporter genes) to provide information on limiting factors (oxygen, nitrogen, phosphorus, and iron availability) in bulk soil and rhizosphere environments.

The available information clearly illustrates the opportunities by the new DNA/RNA-based techniques to describe abundance and diversity of indigenous *Pseudomonas* populations in natural soil and rhizosphere samples. Molecular detection of *Pseudomonas* spp. among environmental 16S rDNA clones confirmed the numerous observations made by cultivation-based methods that *Pseudomonas* spp. are more abundant in rhizosphere than in bulk soil.¹⁶⁵ Smit et al.¹⁶² studied the abundance of *Pseudomonas* in wheat rhizosphere of Dutch agricultural soil and found the gamma-Proteobacteria to comprise 10% and 16% of soil isolates (1/10 TSA medium) and rDNA clones, respectively. The stimulatory effect of the rhizosphere of several crop plants on soil *Pseudomonads* was also shown.¹⁰⁵ The number of clones phylogenetically related to *Pseudomonas* spp. was higher in both *Lolium perenne* (ryegrass) and *Trifolium repens* (white clover) rhizosphere soil compared to bulk soil. Clone sequencing was not performed in this study, but affiliation to *Pseudomonas* sp. was demonstrated by colony hybridization using the *Pseudomonas*-specific PSM_G oligonucleotide probe. In a parallel study by Marilley and Aragno¹⁰⁴ based on partial sequencing of the 16S rDNA clones, the plant roots were shown to have a selective effect toward the gamma-Proteobacteria, leading to a predominance of *Pseudomonas* spp.

Finally, Duineveld et al.⁴⁰ made an interesting attempt to compare the relative abundances of 16S rDNA and corresponding 16S rRNA (after RT-PCR) fragments in *Chrysanthemum* rhizosphere soil. Prominent PCR products were separated by DGGE and excised for sequencing in order to identify both active and total populations as revealed by abundant 16S rRNA and 16S rDNA fragments, respectively. The rDNA analysis demonstrated fewer DGGE bands (lower diversity) in the rhizosphere and *Pseudomonas* spp. were among the abundant genera as judged from the database homologies obtained. Moreover, apart from several *Bacillus*-related bands, at least three out of 12 of the 16S rRNA bands were related to *Pseudomonas* spp. Several *Pseudomonas*-specific primer sets have recently been developed to study the effect of different agricultural management,⁴⁶ influence of plant species (oil seed rape and strawberry), and soil site (Costa, in prep.) on the diversity of *Pseudomonas* communities in soil and rhizosphere.

Compared to phylogenetic diversification, little has yet been done by the DNA/RNA-based techniques to reveal contributions by indigenous *Pseudomonas* spp. populations to functional gene pools and their expression in soil. Where *Pseudomonas* spp. are expected to contribute exclusively or significantly to specific traits and activities in soil, for example, denitrification, degradation of specific xenobiotic compounds, or production of antimicrobial metabolites, direct studies of the functional genes and their expression in natural soil and rhizosphere samples could eventually demonstrate the actual role of this bacterial group *in situ*. In relation to the role of indigenous bacteria in plant protection, interesting candidate genes would be those supporting siderophore or antibiotic (DAPG, phenazine) production, since they are all relatively well described and suitable for studies of diversification and expression.

Finally, the understanding of suppressive soils, where indigenous bacteria support natural control of plant-pathogenic microorganisms, must be improved by analysis of the diversity of microorganisms. In addition to the work by cultivation-based methods, future progress may also here include attempts to identify the functional gene pools involving, for example, a search for the *Pseudomonas* spp.-supported production of antimicrobial metabolites. In this context, the near future will undoubtedly bring the first reports using rapid screening by functional gene arrays, including those from the plant-protecting *Pseudomonas* spp., in natural soil and rhizosphere samples.

6.4 SUMMARY AND PERSPECTIVES

Since the cultivation approach is by far unable to represent the natural diversity of environmental microbes, cultivation-independent techniques based on molecular markers have been successfully used to omit this bias. However, it has to be stated that also these approaches are far away from being free of bias; one example is that PCR is apparently not giving a true blueprint of the natural microbial assemblages. Some sequence types may preferentially be amplified and minor parts of

the community will not be seen because of the inherent sensitivity limits also true for the PCR approaches. Therefore, it is recommended at present to include also PCR-independent molecular methods to get more insight into the diversity of rhizosphere and soil microbes. Using the so-called "cyclic rRNA approach,"³ both identification and localization of yet uncultured or cultured bacteria can be performed *in situ* using sensitive versions of the FISH approach combined with CLSM-analysis. Even in the root environment where probably a higher portion of the microbes are thought to be easily culturable, new species or even genera are continuously discovered, especially if rhizospheres or endorhizospheres of less investigated plants are investigated. In addition, the discovery of the high diversity of bacteria associated with surfaces or even within hyphae and spores of endo- and ectomycorrhizae underscores that both the structural and functional complexity of the rhizosphere is far from being fully understood. However, the available tools for *in situ* functional diversity analysis of rhizosphere microbes are very promising to make further important progress in this endeavor.

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7 Diversity of Biofilms and Their Formation Processes

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Over the last decade, the concept of sessile microorganisms possessing a distinct developmental microbial lifestyle has been fully embraced in the disciplines of both microbiology and ecology. Cells adhere to each other at an interface aided by extracellular polymeric substances and develop into assemblages of microcolonies with more or less confluous architecture referred to as biofilms. While embedded in a biofilm, a cell's phenotypic expression can be altered to a state not commonly found in planktonic cells (Costerton et al. 1995; Prigent-Combaret et al. 1999; Watnick and Kolter 2000). Claude ZoBell's (1943) classic paper exemplifies the infancy stages of biofilm research by focusing on the effect of solid surfaces on microbial activity. ZoBell investigated marine environments, but biofilms are also routinely encountered in other environments, such as the rhizosphere. In this chapter, we begin with a historical account of biofilm research, summarize the current state of knowledge about biofilm formation processes, and discuss the prevalence of biofilms in soil and their role in agricultural systems.

7.1 WHAT CONSTITUTES A BIOFILM?

A useful model definition was developed at the Dahlem-Konferenz in 1984 and states that “a biofilm is a collection of microorganisms and their extracellular products bound to a solid (living or inanimate) surface (termed as substratum)” (Marshall 1984). Since 1984, this definition has been broadened from meaning merely bound to a solid surface to bound at an interphase. Examples of interphases are solid/liquid, liquid/liquid, solid/gas, and liquid/gas. A biofilm comprises the following materials: water, extracellular polymeric substance (EPS) matrix (i.e., slime), and cells (heterogeneous).

Each component of the biofilm serves a unique function, with all components working in concert. The fluid delivers carbon and other essential nutrients to various locations within the

biofilm. The EPS consists of polysaccharides, proteins, and nucleic acids that originate from microbial excretion and lysis. The intercellular spaces of biofilms house EPS whose primary role is thought to supply the bulk of the spatial structure or biofilm architecture (Flemming et al. 2000). The EPS can comprise the majority of the material on a volume and mass basis. The microbial community is very heterogeneous and includes both eukaryotes (e.g., protozoans, fungi, nematodes, etc.) and prokaryotes (Macleod et al. 1990; Jones and Lock 1993; Flora et al. 1995; Sibille et al. 1998; Corbin et al. 2001). In some cases, the substratum can be a source of food, for example, when microbial growth occurs on crystals of polycyclic aromatic hydrocarbons (Wick et al. 2002; Rodrigues et al. 2003).

7.2 ORIGINS OF BIOFILM RESEARCH

The history of biofilm research dates back to the first half of the 20th century, with researchers studying ecology (Henrici 1933; Zobell and Allen 1935), food sciences (Heukelekian and Heller 1940), limnology (Rubentschik et al. 1936; Smith and Zobell 1937), chemistry (Blodgett 1935), and soil sciences (Cholodny 1924, 1930; Allison 1947), all recognizing the existence of biofilms in these various environments.

Under carbon- and nutrient-limited environments, organisms quickly (i.e., within minutes) congregate and form “slimes,” “microbial mats,” “slabs,” or “Aufwuchs,” all of which are synonymous with the modern term “biofilm.” This hypothesis was first surmised by ZoBell and Anderson (1936), who found that organisms have a tendency to flee toward solid surfaces as the nutrient concentration decreases. In addition, Heukelekian and Heller (1940) discovered that *E. coli* only grows under low nutrient conditions (<0.5 to 2.5 mg/L) when solid surfaces are present. These original experiments were confirmed by van Loosdrecht et al. (1990), who found sessile cells to outnumber suspended bacteria by several orders of magnitude under low carbon and nutrient conditions. Organisms existing in a biofilm matrix have an advantage over planktonic cells since they are continually exposed to fresh substrate and nutrients (1) in the aqueous phase that flows past them or (2) sorbed to the substratum itself (Ehlers and Bouwer 1999).

The Russian scientist Cholodony (1924, 1930) devised an effective technique to qualitatively identify the presence of biofilms in the subsurface via glass slides submerged in soil (fig. 7.1). From the days of Cholodony, the bulk of research associated with biofilms in the subsurface has related to the clogging of interstitial pores (Allison 1947; Gupta and Swartzendruber 1962; Avnimelech and Nevo 1964; MacLeod et al. 1988; Vandevivere and Baveye 1992a, 1992b). Indeed, bioclogging of interstitial pores is common in the subsurface, but whether or not the microbes comprise continuous or discontinuous patches of microcolonies is still under debate. The emphasis on clogging has slowly been shifting toward a more thorough understanding of the role of subsurface

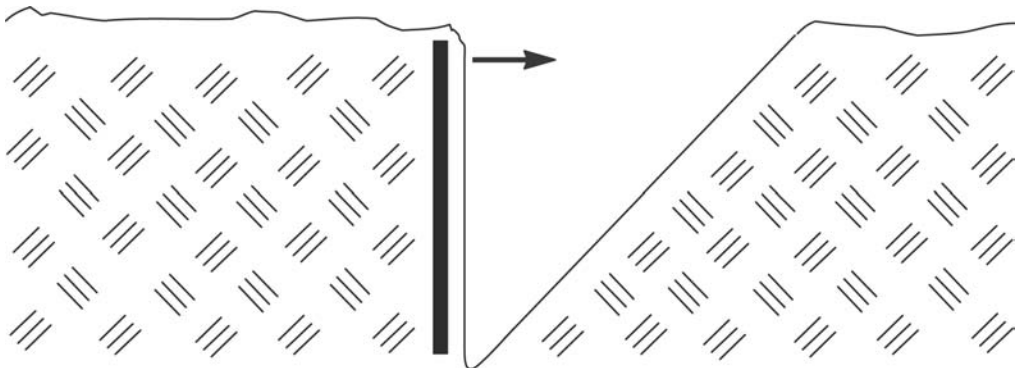


FIGURE 7.1 Glass slide approach developed by Cholodny (1930) (redrawn). The glass slide is represented by the solid vertical rectangle, which is removed from the soil in the direction presented by the arrow.

biofilms in the bioremediation of contaminated aquifers (Wolfaardt et al. 1994; Bouwer et al. 2000; Ebihara and Bishop 2002; Vayenas et al. 2002; Beyenal et al. 2004) coupled with approach(es) to visualize subsurface samples via microscopy (Vayenas et al. 2002).

The pioneers of biofilm research posed the question of why cells adhere to surfaces. ZoBell (1943) confirmed through experiments that the substratum supplies an opportunistic environment for bacteria to sequester molecules of interest. Adhered bacteria are not influenced by the effects of Brownian motion and diffusion that tend to separate cells from their extracellular enzymes and/or hydrolyzates. In fact, the adhered bacterial cell should be able to concentrate extracellular enzymes and hydrolyzates through the interstices at the tangent of the bacterial cell and solid surface(s). It was postulated that as the biofilm thickness increases, more interstitial or capillary spaces form that retard the diffusion of materials away from the cells and favor the absorption of soluble nutrients. In addition, Danielli et al. (1937) surmised that the solid surface facilitates a favorable orientation of bacteria extracellular enzymes.

The original works performed by the authors listed above and various others posed many of the questions that are finally being answered in this day and age with the advent of molecular biological tools, such as fluorescent *in situ* hybridization (FISH) probes, confocal laser scanning microscopy (CLSM), and microelectrodes. Over the past 30 years, the utilization of such tools has led to research encompassing a wide array of biofilm environments, including alpine streams (Geesey et al. 1978), rumen (Cheng et al. 1981), industrial pipelines (Van der Wende et al. 1989), marine environments (Fletcher 1986; Decho and Lopez 1993; Dalton et al. 1996), porous rock (Haldeman et al. 1994), rhizosphere (Briones et al. 2002), and infected medical devices (Gristina and Costerton 1985).

7.3 ANALYTICAL TOOLS TO INVESTIGATE BIOFILMS

The earliest biofilm communities examined via microscopy were referred to as “Aufwuchs” by German microscopists, and primarily dealt with sessile protozoans and metazoans (Wimpenny et al. 2000). ZoBell (1935, 1943) initiated a systematic approach to observing bacteria on surfaces, spawning the development of new tools for *in situ*, *in vitro*, and *in vivo* examination of biofilms.

Over the past few decades, several analytical tools have been developed to augment the advancement of biofilm insight. Epifluorescence microscopy has been used widely in microbiology, providing information related to microbial activity, total cell counts, and the two-dimensional distribution of microorganisms (Wolf et al. 2002). The combination of epifluorescence microscopy with other techniques, such as those investigating nucleic acids, provides a unique opportunity for obtaining insights into the dynamics and distribution of microbial populations in biofilms.

The application of the conventional scanning electron microscope (CSEM) to biofilm studies in the early 1970s and 1980s produced images of the complex matrix comprising a biofilm (Hirsch and Pankratz 1970; Eighmy et al. 1983). Although an invasive technique, CSEM images supplied researchers with useful information on biofilm surface structure (Wimpenny et al. 2000). The dilemma with invasive procedures is that they do not supply representative results because of artifacts produced during the dehydration and preparation steps of specimens. The artifacts associated with CSEM are alleviated through several advancements in microscopy, including environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM) (Surman et al. 1996). ESEM relies upon surrounding the sample with a gaseous environment to ensure that a conductive milieu is present (Little et al. 1991). This approach enables one to monitor organisms under pseudo-natural conditions and circumvents the laborious dehydration steps of CSEM.

The advent of confocal laser scanning microscopy (CLSM) for analyzing biofilm structural heterogeneity greatly increased the understanding of biofilm architecture over the past decade (Wolf et al. 2002). This microscopic technique is based on the same principles as epifluorescent microscopy, but it is unique in that it uses a pinhole or multiphoton microscopy to filter light so that only

the focused light emitted from a plane of interest is detected. The instrument scans the sample and generates a clear, high-quality planar image of the physical structure of the biofilm. The scanned optical sections of the biofilm are stacked for a 3-D topographical view of the biofilm (Wimpenny and Colasanti 1997). CLSM has been used along with molecular biological techniques to detect and identify specific individual cells. Insights into biofilm spatial structure (the architecture) using CLSM support the hypothesis that a biofilm is a complex, heterogeneous environment.

Molecular biological techniques have facilitated noninvasive analysis of complex microbial communities. A mere ~10% of the organisms available in nature are presently amenable to cultivation (Amann et al. 1991). Furthermore, culture-dependent environments potentially impose different environmental conditions than the natural state and lead to environmental biases (Dunbar et al. 1997). Ribosomal RNA is present in all organisms and contains conserved and variable regions that are specific to certain organisms; this form of nucleic acid is an ideal target for oligonucleotide probes to aid in identification of microbes (Wolf et al. 2002). The technique most frequently used in biofilm analysis, fluorescent *in situ* hybridization (FISH) of whole cells, enables both qualitative and quantitative analysis. FISH is especially useful because it can be used to find organisms that are either not cultivable or difficult to cultivate in the laboratory (Wimpenny et al. 2000). When one is interested in verifying the presence of specific organisms, FISH offers an advantage over general nucleic acid stains such as DAPI, the SYTO family, and propidium iodide since FISH involves probes of varying degrees of specificity for identification of microbial taxons, such as genera or species.

The combination of FISH with CLSM generates information regarding the spatial heterogeneity and microbial diversity within a biofilm over time. FISH probes range in specificity from domain (EUB338 series probe set) to species. PCR involving amplification of specific target sequences, followed by cloning of rRNA genes, is used prior to FISH to provide sequences for the design of suitable oligonucleotide probes. Upon knowledge of the phylogenetic diversity of an environmental sample, appropriate FISH probes are designed to detect the complementary rRNA in whole cells.

As with other analytical techniques, limitations exist for FISH. The sample must be homogenized to aid penetration of the probe, which hinders locating species within flocs/biofilms. Also, an abundance of rRNA must be produced per organism present or else the values will be underestimated. And finally, the major limitation in using FISH relates to function. Even though FISH presents valuable quantitative information with regard to location and concentration, it does not supply information relating to function (i.e., substrate uptake). Resolution of the functionality issue can be achieved via microautoradiography (MAR) (Lee et al. 1999). Microautoradiography tracks the uptake of radiolabeled substrate and can work in concert with FISH and CLSM to correlate structure and function of organisms present in biofilm microbial communities (Lee et al. 1999). To date, MAR has not been applied to biofilms in soil. CLSM, FISH, and MAR lead to insights into community structure, spatial heterogeneity, and spatial functionality.

Microsensor probes measure *in situ* chemical gradients, such as nitrate, nitrite, pH, sulfate, oxygen, etc. (de Beer and Vandenheuvel 1988; de Beer and Sweerts 1989; de Beer 1999). Microsensors rely upon a probe that is inserted into the biofilm normal to the substratum, with subsequent chemical measurements taken at predefined biofilm depths. The use of microsensors confirmed the postulation that chemical profiles exist in biofilms, and microniches prevail within a complex biofilm matrix. The majority of the research utilizing microsensors is in aqueous biofilm environments, such as water distribution systems and lake sediments. Although these environments differ considerably from soil environments, the previous and current research in these areas present ideas with regard to chemical gradients that can be compared to the soil matrix. The major limitation of microsensors is that chemical profiles are produced only at the measured location. To alleviate this concern, several microsensors should be utilized in concert to comprehend the complexity of the biofilm (Yu et al. 2004).

The modern analytical techniques addressed thus far have greatly advanced knowledge of biofilms. Thus far, soil community population dynamics have mostly been studied without regard to biofilms by fingerprinting methods like denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Torsvik and Øvreås 2002). Metagenomics offer a new opportunity to obtain PCR-independent diversity and gene expression-related information on complex communities. The term “metagenome” is derived from the idea that the combined genomes of all soil microbes can be considered one large soil microbial community (Rondon et al. 2000). Metagenomics is based on the extraction of DNA from samples, cloning the DNA in large pieces into a readily culturable organism such as the commonly used *E. coli*, and screening clones for biological activity (Handelsman et al. 1998). Although metagenomic research is still relatively new, the data agree with previously obtained data on genetic diversity, in that soil contains extreme microbial diversity. The key component still lacking from our knowledge database relates to the paucity of information on PCR-independent total diversity and gene expression of microbes in soil, especially within biofilms.

7.4 HIGHLY STRUCTURED BIOFILM COMMUNITIES

The implementation of modern analytical tools has yielded an exorbitant amount of information and insight into biofilm formation (Stoodley et al. 2002), function (Lee et al. 1999), gene expression (Prigent-Combaret et al. 1999), architecture (Heydorn et al. 2000), horizontal gene transfer (Hendrickx et al. 2003; Molin and Tolker-Nielsen 2003; Hendrickx and Wurtz 2004), microbial spatial dynamics (Fukui et al. 1994; Unge and Jansson 2001), and communication between cells (Sauer et al. 2002; Wurtz et al. 2004). A comprehensive explanation of biofilm formation is undertaken in the next section. Although biofilm research has expanded at an exponential rate, we have only begun to decipher the complex system of environmental cues and phenotypic responses that shape and form biofilms (Stoodley et al. 2002).

Insight gained from microscopic experiments established that biofilms are comprised of highly structured, matrix-enclosed communities (Costerton and Stewart 2001; Stoodley et al. 2002) whose cells undergo gene expression patterns profoundly different from planktonic cells (Sauer et al. 2002; Stoodley et al. 2002). The realization that microbes embedded in the EPS matrix have contact with moisture, carbon, electron donors, nutrients, etc., was first elucidated with the aid of CLSM (Lawrence et al. 1991). The CLSM images fostered the notion that channels and void spaces may exist within the complex matrix, ensuring that microbes spatially located next to the substratum have access to moisture, carbon, electron donors, nutrients, etc. (fig. 7.2).

The development of a biofilm enables the proliferation of bacteria in a wide range of environments and also fosters phenotypic expression not commonly found in single cells (Costerton et al. 1995; Prigent-Combaret et al. 1999; Watnick and Kolter 2000). This is due to environmental conditions encountered in the biofilm that differ markedly from the bulk fluid environment. The biofilm matrix induces an environment with sharp chemical gradients, promoting the establishment of micro-niches such as anaerobic conditions for strict anaerobes (de Beer 1999). Obligate anaerobes located deep within the biofilm have the ability to survive despite predominantly oxic conditions in the bulk fluid. Furthermore, biofilms protect cells from dessication, UV, predation, and bactericides (Costerton et al. 1995; Davey and O'Toole 2000).

The biofilm matrix functions as a consortium cooperating in a complex but coordinated manner (Caldwell 1995; Costerton et al. 1995; Davey and O'Toole 2000). The biofilm environment creates an opportunistic medium for horizontal gene transfer, synergistic interactions, and quorum sensing. The EPS matrix induces conditions favorable for horizontal gene transfer (Wurtz 2002). Gene transfer is desired since it increases metabolic capability and enhances adaptability to changes in environmental conditions. Cells embedded in the biofilm matrix are more susceptible to the three primary means of horizontal gene transfer (conjugation, transduction, and transformation) due to extended cell-to-cell contact time coupled with the sheltering and adhesion of free DNA and viruses.

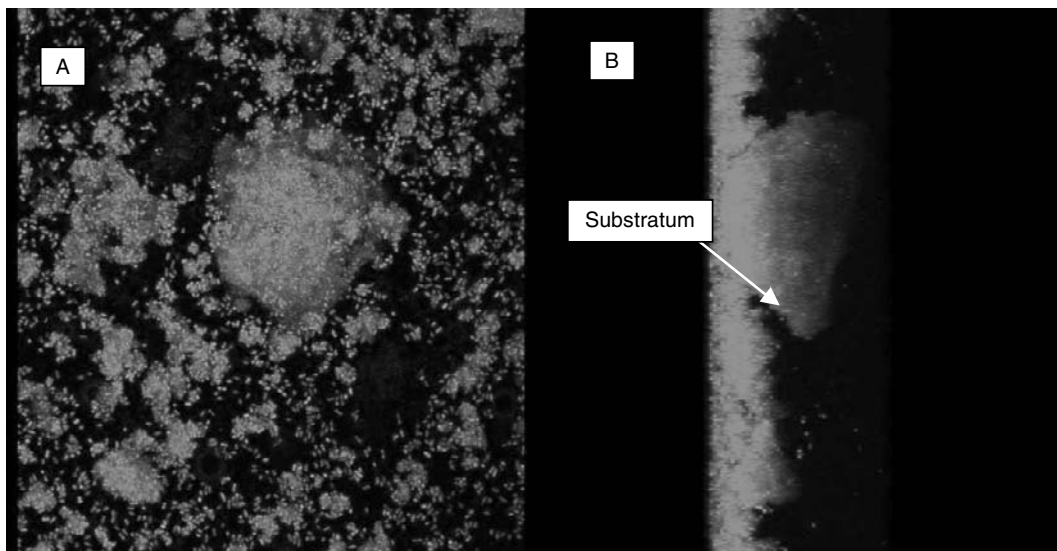


FIGURE 7.2 CLSM images depicting the void spaces and spatial heterogeneity of an *Acinetobacter* sp. BD413 pure culture stained with Syto 16 from (A) the aerial view and (B) the planar view. Source: Robin Gray Merod.

The micro-niches indicative of biofilms facilitate metabolic reactions that are not necessarily feasible while cells are in the planktonic state. Due to the close proximity of cells and immobile conditions associated with the biofilm matrix, comensalistic, synergistic, and amensalistic microbial interactions ensue within the biofilm. During quorum signaling, cell-to-cell communication is feasible via cell-density dependent diffusible molecules, such as acyl homoserine lactones. These quorum signals may accumulate within the complex biofilm matrix (Costerton et al. 1995; Davey and O'Toole 2000). At the time of writing there is no unequivocal evidence that quorum sensing is required for early stages of biofilm formation (Heydorn et al. 2002; Beloin et al. 2004). Rather, environmental factors may be the dominant forces affecting biofilm architecture. Some evidence suggests that redox conditions can modulate biofilm development via quorum sensing (Yoon et al. 2002).

7.5 FORMATION AND DEVELOPMENT OF BIOFILMS IN SOIL

The formation of biofilms is initiated when planktonic cells adhere to the substratum, coupled with de novo EPS production. The distinct time frame during which cells aggregate and adhere onto a substratum and become a biofilm is variable and there is no agreement as to how many cells must be present. The transport of bacteria in porous media is typically governed by aqueous phase convective movement, along with retardation by adhesion onto surfaces and sieving or trapping in interstitial pores (Ehlers and Bouwer 1999). As originally postulated in the early half of the 1900s, the adhering capacity of organisms increases with time (Blodgett 1935). The adhesion relates to a wide array of physicochemical properties of both the substratum and cells. From a morphological perspective, whether a cell is gram negative or positive will not dictate adhesion feasibility (Rubentschik et al. 1936). An additional tendency deals with whether or not the cell is undergoing starvation. Short-term starvation enhances adhesion (Dawson et al. 1981; Kjelleberg 1984), whereas long-term starvation might enhance bacterial transport through porous media (Lappin-Scott et al. 1988a, 1988b; MacLeod et al. 1988). The rationale behind the statement stems from the notion that cells undergoing short-term starvation will settle and produce substances, if necessary, to offset

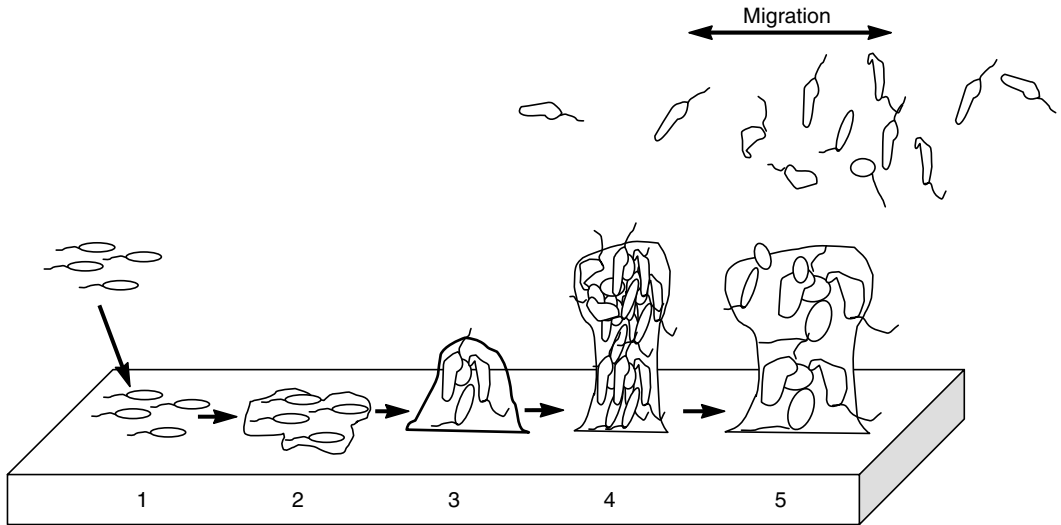


FIGURE 7.3 Diagram illustrating the various stages of biofilm formation: (1) attachment of cells to substratum, (2) production of EPS, (3) early phases of biofilm architecture, (4) maturation of biofilm architecture, and (5) dispersion of cells. Adapted from Stoodley et al. (2002).

starvation, whereas cells subjected to long-term starvation might require a different environment to ensure cell maintenance energy.

Once cells adhere to a substratum, biofilm formation can occur, and the steps involved in this process have been modeled conceptually (Stoodley et al. 2002): (1) adhesion of cells to substratum, (2) EPS production yielding irreversible attachment, (3) preliminary stages of biofilm architecture, (4) developed mature biofilm architecture, and (5) the dispersion of single cells from the biofilm. figure 7.3 illustrates the stages of each step during the formation process. The biological mechanism for detachment in stage 5 is not well understood. Physical forces leading to detachment may play a much larger role in soil environments via erosion and abrasion. A factor not considered in the model that may play a considerable role in the soil environment is predator grazing.

The first step, adhesion of cells to substratum, is governed by physicochemical properties coupled with microbial properties (Busscher et al. 1995; Oliveira et al. 2003). Predicting the adhesion affinity between an organism and substratum is ascertainable to a certain degree by way of thermodynamics. The change in free energy arises through a combination of DLVO interactions, polar interactions, Brownian motion, and EPS production (van Oss et al. 1988; van Oss 1994). The cells are supplied via at least three routes (Stoodley et al. 2002): (1) redistributed attached cells (Korber et al. 1995; Dalton et al. 1996), (2) binary division of attached cells (Heydorn et al. 2000), and (3) the transition of cells from a planktonic to a sessile state (Tolker-Nielsen et al. 2000).

Upon adhesion, EPS production ensues and results in adhered “irreversible” attachment (Oliveira et al. 2003). For this to occur, the organisms must maintain contact with the substratum and grow (Stoodley et al. 2002). ZoBell (1943) was the first to notice this transition from reversible to irreversible and Characklis (1990) characterized the transition. The EPS is comprised of polysaccharides, proteins, and nucleic acids that provide structural support for the biofilm (Brewer et al. 1991). Several biological factors, such as twitching motility, growth rate, cell signaling, EPS production, environmental conditions, nutrients, flow regime, etc., play roles in dictating the shape and function of biofilms. A compilation of these variables, plus strong hydrodynamic influences such as shear forces, leads to the maturation of biofilm architecture.

The EPS traps cells within a matrix at high densities and influences the morphology, architecture, coherence, physiochemical properties, and biochemical activity of the matrix (Wingender et al.

1999; Flemming and Wingender 2001). Chemically, the EPS is the least understood of the biofilm components, which relates to the difficulty in extracting EPS from a biofilm matrix (Wuertz et al. 2001). Low extraction efficiencies pose a challenge when attempting to resolve the composition of EPS, as well as its functional role in determining architecture. Advancement of EPS knowledge might facilitate one's ability to adequately predict biofilm performance. The rationale behind this statement stems from the fact that EPS may comprise upwards of 98% of the total biomass on a wet basis (Flemming 1995; Lazarova and Manem 1995; Zhang and Bouwer 1997; Jahn and Nielsen 1998; Zhang et al. 1998a, 1998b, 1999). From a soil vantage point, the difficulties involved in EPS extraction are not well understood. An extraction procedure will destroy both biofilm and soil structure. Fluorescently labeled lectins have been used to describe EPS *in situ* in defined biofilms (Johnsen et al. 2000) and natural biofilms (Neu et al. 2001), but not in soil. Due to the lack of *in situ* approaches for soil, the majority of biofilm research utilizes the soil's saturated hydraulic capacity as an indicator of EPS production.

It is suggested that architecture is the defining feature in biofilm matrices, but the mechanisms controlling architecture are not fully understood (Hermanowicz 2003). Deciphering these architectural attributes will enable one to comprehend the spatial dynamics of chemicals and individual organisms, the metabolic processes, the fluid and biofilm interactions, and the rigidity of the biofilm structure (Zhang et al. 1998a, 1998b, 1999).

Detachment of excess biomass ensues as external forces exceed the internal strength within the biofilm (Morgenroth 2003). Knowledge of the fundamental mechanisms controlling detachment is still in its infancy. The various mechanisms of detachment include erosion, abrasion, sloughing, predator grazing, and biofilm-controlled detachment of cells. Although these five mechanisms can be differentiated from one another, a mathematical model that effectively describes detachment is still missing. Within the first four types of detachment, different modes of detachment exist (i.e., surface detachment, volume detachment, and dynamic detachment), that are thought to mathematically predict detachment over time (Saez and Rittmann 1992; Morgenroth and Wilderer 2000; Morgenroth 2003). Determining the primary mode of detachment is crucial in predicting cell residence time, population dynamics, and microbial ecology (Morgenroth 2003). The fifth mechanism, biofilm-controlled detachment, is commonly referred to as "willful" detachment of cells. Furthermore, cells have been shown to migrate as a unit, in both the ambient flow direction (Stoodley et al. 1999a, 1999b) and in the direction opposite the ambient flow (Venugopalan et al. 2005). These data suggest that the migration of microcolonies is not necessarily induced by flow, but is an inherent biofilm property (Venugopalan et al. 2005).

7.6 PREVALENCE AND DIVERSITY OF BIOFILMS IN SOIL

It is generally understood that microorganisms located in the highly structured soil environment are subjected to a wide array of stress factors, especially nutrient limitations or fluctuations (van Elsas and van Overbeek 1993). Within soil environments, organisms are subjected to the three phases that make up soil: solid, liquid, and gaseous phases. The distribution of nutrients within these three phases is extremely heterogeneous (Kilbertus 1980; Smiles 1988). Although organisms in soil experience stressful nutrient availability, a more than adequate concentration of carbon and nutrients exists to promote the growth of organisms in most environments (Williams 1985). However, the nutrients necessary to facilitate the growth of organisms in soil are not readily available for chemical and/or biological reactions; that is, they are not bioavailable (Hamelink et al. 1994).

Does the lack of bioavailable substrate lead to the formation of biofilms in soil? Answering such a question requires extensive laboratory scale testing along with subsequent field studies. Currently, a noninvasive approach that does not compromise the soil structural environment is lacking. The use of epifluorescence microscopy and CLSM has been limited because of high autofluorescence in the soil matrix. Various authors have attempted other approaches that investigate aspects of flow, such as using hydraulic conductivity, to act as an indicator of bioclogging (Allison

1947; Avnimelech and Nevo 1964; Mitchell and Nevo 1964; Shaw et al. 1985). The data suggest bioclogging is an issue within soil.

Several researchers hypothesize that the high specific surface area indicative of aquifer porous media yields bacteria attached to the soil solids (Harvey et al. 1984; Balkwill 1988; Kolbel-Boelke et al. 1988), whereas others hypothesize that this high surface area yields a small fraction of soil colonization (Vandevivere and Baveye 1992b, 1992a; Bouwer et al. 2000). The sorbed bacteria have been thought to play a key role in the biodegradation of contaminants and clogging of porous media (Raleigh and Flock 1965; Vecchioli et al. 1975; Oberdorfer and Peterson 1985; Wetzel et al. 1986; Staps 1990). However, it is estimated that biofilms in the subsurface comprise sparse, isolated colonies with cell concentrations ranging from 10^5 to 10^7 cfu/g of soil (table 7.1) (Bouwer et al. 2000). Based on actual cell numbers, it is questionable whether biofilms do form in the subsurface. Although the data do not at this point in time resolve the debate, several models have attempted to use both viewpoints to illustrate the role of biofilms in soil matrices.

The continuous biofilm school of thought achieved excellent correlation between expected permeability loss and losses measured while modeling biofilter growth in porous media (Taylor and Jaffe 1990a, 1990b; Cunningham et al. 1991). Other authors question the mere existence of biofilms in the subsurface (Vandevivere and Baveye 1992a, 1992b.) The argument opposing the presence of biofilms in the subsurface relates to isolated bacterial aggregates in the subsurface. Depending on substrate bioavailability, microbial cell accumulation will develop into either patchy, discontinuous colonies or merged colonies forming a continuous film. Rittmann (1993) addressed the importance of biofilms in modeling the subsurface and how the influence of biofilms hinges on the modeling goal(s). A modeler interested in substrate removal should not be concerned with whether adhered cells are continuous or discontinuous biofilms, whereas a modeler concerned with spatial distribution of attached biomass and permeability needs to distinguish between continuous and discontinuous biofilms.

Vandevivere and Baveye (1992a) made an attempt to resolve the debate on whether biofilms or discontinuous patchy microcolonies reduce hydraulic conductivity in porous media. Four different strains, including one EPS producer, were inoculated into four separate laboratory scale columns. Of the four strains, only the EPS-producing strain significantly impacted the saturated hydraulic conductivity, HC_{sat} . However, the EPS that negatively affected HC_{sat} was characterized as being “loose” slime and not the cell-bound EPS associated with a biofilm. The authors did not attribute the decrease in HC_{sat} to an increase in population densities since the glucose consumption was comparable from column to column. Their data suggest that HC_{sat} decreased from EPS, hindering the transport of fluid. The results were verified through CSEM imaging of core samples, which verified the presence of a “long fibrillar meshwork” of EPS only in the column containing the EPS-producing strain. Furthermore, the production of EPS did not affect cell multiplication or cell movement within the soil columns (Vandevivere and Baveye 1992a).

TABLE 7.1
Distribution of Microorganisms in Water and in the
Subsurface Environment

	Cells/gram solids (CFU/mL)	Surface coverage (%)
Water column	10^6 – 10^8	N/A
Sediments	10^8 – 10^{10}	0.08%–8%
Shallow aquifer	10^5 – 10^8	0.00008%–0.08%
Deep aquifer	10^4 – 10^6	0.000008%–0.0008%

Adapted from Bouwer et al. (2000).

The research mentioned above did not resolve the debate on existence of biofilms within soil and the subsurface, but it was pivotal in recognizing the role of EPS in experiments and models for a soil environment. The current debate revolves around how many cells constitute a biofilm. An additional aspect of Vandevivere and Baveye's work is that not all cells showed the ability to produce EPS, leading to the question: Do cells have the ability to alter their gene expression *in situ* to produce EPS? Recent molecular data from single-species biofilms suggest this is possible. Regardless of a cell's ability to produce EPS, both prokaryotes and eukaryotes have been found in biofilms. Do cells become entangled within the EPS web-like matrix, and if entangled, do they become active players in the complex environment referred to as a biofilm? We speculate that regardless of whether or not a cell is an EPS producer, all microbes play a role in the complex soil biofilm ecology.

The diversity of biofilms in soil is a relatively new and upcoming field in soil sciences. The advent of metagenomic sciences has created a new epoch for biological investigations (Torsvik and Øvreås 2002). Early metagenomic studies have confirmed the previous hypotheses on the complexity and diversity developed with the aid of culture-dependent and culture-independent techniques (Torsvik and Øvreås 2002). As previously addressed, the microbial community associated with biofilms derived from culture-independent approaches has been heterogeneous and includes both eukaryotes (e.g., protozoans, fungi, nematodes, etc.) and prokaryotes (Macleod et al. 1990; Jones and Lock 1993; Flora et al. 1995; Sibille et al. 1998; Corbin et al. 2001). Thus far for metagenomic studies (Rondon et al. 2000), soil systems and biofilms in drinking water distribution systems (Schmeisser et al. 2003) are the most closely related models available for describing the diversity of microbes in biofilms. Within soil systems, the metagenomic clone database from agricultural soil in Madison, Wisconsin, contained DNA from a wide range of phyla, including sequences of low-G+C, gram-positive *Acidobacterium*, *Cytophagales*, and *Proteobacteria* (Rondon et al. 2000). Schmeisser et al. (2003) postulated that since drinking water distribution samples contain fewer bacterial species than soil samples, these systems are ideal models for studying metagenomes. The majority of the clones within the metagenomic database seemed to be closely related to the *Proteobacteria*, with a small fraction devoted to *Actinobacteria*, low-G+C gram positives, and the *Cytophaga-Flavobacterium-Bacteroides* group (Schmeisser et al. 2003). Although neither study referred to biofilms in soil, the results are promising in confirming the power of the metagenomic approach in identifying the organisms responsible for diversity.

7.7 PREVALENCE AND DIVERSITY OF BIOFILMS IN THE RHIZOSPHERE FOR AGRICULTURAL SYSTEMS

The primary difference between organisms in the bulk soil and rhizosphere soil is carbon and nutrient availability. Plant roots supply one of the major sites of carbon input into soil (van Elsas and van Overbeek 1993). Between 5 and 50% of carbon comprising the standing root may conceivably end up as rhizodeposits (Newman 1985; Trofymow et al. 1987). The estimated ratio between the amount of soluble organic carbon in the rhizosphere and bulk soil will range from 3 to 30 (Atkinson 1990). This carbon acts as a vector to support a rich, active rhizosphere microflora (Campbell and Greaves 1990). Additionally, the carbon released by roots is less recalcitrant and labile than carbon found in the bulk soil (van Elsas and van Overbeek 1993).

Rhizodeposits of labile carbon have been found to generally form the patchy discontinuous nonuniform colonies discussed in the previous section on biofilms in soil. The research on biofilms within the rhizosphere of agricultural systems dates back to the 1970s, with emphasis on wheat roots (Rovira and Campbell 1974). Rovira and Campbell (1974) propose biofilm location to be directly linked to the root exudates, since this is the location of released carbon from the plants. Biofilms play a pressing role in rhizosphere proliferation via essential nutrient(s) supply to plants. In the rhizosphere, vital plant-microorganism symbioses prevail, such as those between rhizobia

and leguminous plants (Werner 1992) and the mycorrhizae (Abbott and Gazey 1994; Bowen 1994). Outside of this true symbiotic relationship, researchers are starting to unveil additional relationships between plants and microorganisms. For example, *Pseudomonas* spp. have been found to potentially act as biological control agents to fend off various pathogens (Dandurand et al. 1997; Bloembergen et al. 2000; Bianciotto et al. 2001; Morris and Monier 2003). Additionally, a less direct association that enhances plant growth is referred to as growth-promoting rhizobacteria (Fukui et al. 1994; Kloepper 1994; Wiehe et al. 1996; Morris and Monier 2003). A few examples of growth-promoting bacteria include *Enterobacter agglomerans*, which colonize roots by symplasmata formation (Achouak et al. 1994), bacteria linked with ectomycorrhizae (Sarand et al. 1998), cyanobacteria (Toledo et al. 1995), and *Azospirillum* spp. (Schloter et al. 1993), both involved in nitrogen fixation, phytohormone producers (Hartmann et al. 1983; Lebuhn and Hartmann 1994), and ammonia-oxidizing bacteria associated with paddy rice roots (Briones et al. 2002). FISH probes indicated that the concentration of ammonia-oxidizing bacteria was roughly 2–3 orders of magnitude greater than that of ammonia-oxidizers typically encountered in soils (Briones et al. 2002). Regardless of the agricultural application, EPS production in the rhizosphere leads to a remarkably improved soil structure that enables a stress-free situation with regard to water (Amellal et al. 1998; Alami et al. 1999; Bezzate et al. 2000; Morris and Monier 2003).

The biofilms in the rhizosphere not only enhance nutrient supply and protection from pathogens, but they also improve plant biology. Exhaustive studies have been performed on the occurrence and prevalence of iron plaques on plant roots that may account for upwards of 10% plant dry weight (Hansel et al. 2001; Morris and Monier 2003). Hansel et al. (2001) suggest that the iron oxidation is in part due to biofilms on the roots that release oxygen into an otherwise anaerobic environment. It is suggested that ammonia-oxidizing bacteria acquire oxygen via its host, which in turn acquires a usable form of nitrogen from the nitrifiers (Briones et al. 2002).

7.8 ADDITIONAL AREAS OF BIOFILM INTEREST IN AGRICULTURAL SYSTEMS

Other areas of interest for agricultural systems include the phyllosphere. The wide range of moisture levels encountered globally leads to the complexity of describing this environment (Morris and Monier 2003). The prevalence of biofilms at the phyllosphere has been found to be inferior from a density standpoint compared to those of water-unsaturated systems (e.g., rhizosphere) (Holden et al. 1997; Auerbach et al. 2000; Morris and Monier 2003). Regardless of the sheer numbers, one should not disregard cell density at this interface but take into account interface functionality (Morris and Monier 2003). An additional area of biofilm studies on plants suffering from a paucity of information is biofilms within plant tissues. Assumptions can be made about similar environments that plant tissue biofilms emulate, but the physicochemical nature of these interfaces awaits exploration.

The biofilm assemblages on leaf surfaces have been found to harbor both gram positive and gram negative bacteria, yeast, and filamentous fungi (Morris et al. 1997; Morris and Monier 2003). Microscopic approaches have shown biofilms to be upwards of 100 microns in length, including those found on vegetable crops, herbs, and trees (Morris et al. 1997; Morris and Monier 2003). Several studies have indicated that bacterial aggregates constitute between 30 and 80% of the total bacterial populations on leaf surfaces (Morris et al. 1997; Jacques et al. 2000; Morris et al. 2002; Boureau et al. 2003; Morris and Monier 2003). Normander et al. (1998) found biofilms in the phyllosphere to promote horizontal gene transfer among bacterial species.

7.9 CONCLUSIONS

In summary, research studying the complex matrix known as a biofilm has revealed several key attributes of the biofilm lifestyle. The early trends and ideas developed by the pioneers of biofilm

research are finally being addressed with the advent of molecular biological techniques coupled with advances in microscopy. Although these advancements in methodologies have yielded a voluminous amount of knowledge over the past two decades, researchers have only begun to understand the complex intricacies associated within a biofilm community. Several debates still exist within the scientific community as to even the mere existence of biofilms in soil, how many cells constitute a biofilm, along with several other issues. Regardless of one's position on the issues, the research suggests that biofilms play a viable role in soil agricultural systems.

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8 Diversity of Soil Fungi

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8.1 WHAT ARE SOIL FUNGI?

Common genera of soil fungi include taxa from the Ascomycotina (mostly anamorphic forms) and Zygomycotina (e.g., *Absidia*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Mortierella*, *Mucor*, and *Penicillium*). Species of these genera are commonly isolated by soil dilution techniques as they can produce high numbers of spores and the probability for retrieval is very high. Other taxa mainly grow as mycelium in soil (e.g., *Rhizoctonia* spp.), thus soil-washing techniques are by far more suited to isolate such species, as the spores can be removed from soil or organic particles by repeated washing procedures. Moreover, both arbuscular mycorrhiza (AM) and ectomycorrhiza (EM) are extremely common in soil, but are very difficult or even impossible to quantify by cultivation-based techniques. The application of the term “soil fungi” in a wider sense would also include the saprophytic basidiomycetes (cap fungi, decomposer fungi), as their mycelia colonize the plant debris in the top soil layer.

The present chapter focuses on the wide spectrum of saprophytic microfungi in soil, mostly occurring in their anamorphic form.

8.2 INTRODUCTION

Apart from remote environments such as volcanic vents on the ocean floor and lakes under Antarctica’s ice, the top layers of soil are, according to some ecologists, the most poorly researched habitats on Earth (Copley 2000). In recent years terrestrial ecologists have begun to understand that soil biodiversity is a crucial factor in ecosystems regulation. The most important interactions between

plants themselves, plants with symbionts (fungi and bacteria), and decomposer organisms take place below, respectively, on ground. The interdependency between biodiversity of the different groups of organisms in soil and its impact on ecosystem function is still poorly understood. Consequently, nobody can estimate how soil biodiversity and hence the function of terrestrial ecosystems will be affected by land use in agricultural “ecosystems.” Therefore, large efforts should be taken to describe the system as accurately as possible using all methods available. In recent years, the classical methods of assessing species diversity via culture-based techniques have been replaced by molecular-based techniques to also assess genetic diversity. These are appropriate tools to assess the genetic diversity of certain taxa, but the fidelity to characterize species diversity depends on the quality of sequence databases and the quantity of DNA sequences available for comparison.

The accuracy of a biodiversity index basically depends on correct identification. Correct identification depends on a more or less stable systematics and on the availability of specialists (Vishniac 1996). Due to changes in nomenclature it may sometimes be difficult to trace the identification given for an hyphomycete down in early reports to a more recent equivalent. Taxonomists document the world’s biodiversity by nomenclatural matters (Hawksworth 1991). This perspective is mainly based on the assessment of “species diversity.” Based on the traditional biodiversity scheme, “genetic diversity” and “system diversity” have to be considered likewise. In addition, the concept of biodiversity must be approached from a functional point of view, leading to “functional diversity” as a measure of ecosystem performance (Setälä et al. 1998).

Data on the worldwide distribution of soil-fungi and their specific habitats are vast and have been summarized almost 25 years ago by Domsch et al. (1980). This monograph of soil-borne fungi is still unique and can serve as an excellent identification handbook exhibiting detailed information on ecological and physiological aspects of microfungi. Data were based on inventories or extracted from ecological experiments with single species and their interactions with either microbial or invertebrate pathogens. Thus, ecological data on diversity and species richness are mostly derived from studies in phytopathology, and were mostly pathogen oriented. To the contrary, studies on the biodiversity of microfungi (hyphomycetes) in agricultural ecosystems are comparably rare. In recent years studies have more and more considered the interrelationship of several organisms on different trophic levels.

Attempts at evaluating taxonomic diversity is problematic if the taxonomic information is not integrated in the ecological function (Zak and Visser 1996). An important aspect of fungal biodiversity that has not been addressed adequately is the impact of the fungal growth form on estimates of biodiversity. The fungi are physiologically active when they grow by their mycelium. When biodiversity is estimated by plate counts, the number of colon-forming units (CFU) can be relatively low as colonies grow out from mycelial fragments, but these fungi can be physiologically active and thus functionally active. On the other hand, extensively sporulating species such as *Aspergilli* and *Penicilli* can be functionally inactive, but may occur in high numbers of CFU.

In view of the functionality of soil for agriculture, innovative approaches to study biodiversity should combine classical microbiological, biochemical, and molecular techniques. On the one hand, the diversity of methods applied (especially molecular techniques) has clearly increased, but on the other hand, the classical cultivation-based methods have been neglected, although they have mostly resulted in much bigger species lists compared to molecular approaches. One reason for this is certainly that trained mycologists with the skill to reliably identify a wide spectrum of taxa have become extremely rare. Another reason must be seen in the fact that inventories aiming at the identification of large numbers of isolates require sufficient time, which is extremely costly.

8.3 ECOLOGICAL ASPECTS OF FUNGAL DIVERSITY

8.3.1 DIVERSITY OF ARBUSCULAR MYCORRHIZA: A MODEL FOR THE SAPROPHYTES?

A wide range of investigations on microfungi in soil focused on AM (arbuscular mycorrhizal fungi), as these are directly associated with the plant roots, and thus have direct influence on the nutrition

of plants. The major part of plant species are mycorrhizal, with around two-thirds forming arbuscular mycorrhizal associations (Trappe 1987; Daniell et al. 1999) with fungi of the order Glomales (*Zygomycotina*). The other one-third belongs to the ectomycorrhiza with fungi of the *Basidiomycota* and a few species in the *Ascomycota*. Nonmycorrhizal plants are unexpectedly rare and were estimated to account for approximately 5% (Fitter and Moyersoen 1996). The order *Glomales* hosts about 150 described taxa, which form AM associations with approximately 200,000 plant species and therefore were initially regarded as nonspecific. The fact that plant communities are typically associated with a number of 10–20 AM fungal taxa suggests that different taxa play distinct roles in the symbiosis (multifunctionality) and that fungal taxa may be associated preferentially with particular plant taxa (Daniell et al. 1999). Indeed, the data by van der Heijden et al. (1998) give evidence that there is a high degree of selectivity in natural mycorrhizal communities. Regarding the AM, fungal diversity in agricultural production systems seems to be much lower compared to natural environments. Helgason et al. (1998) showed that fungal diversity in the root system in an arable field was very low (a single taxon), whereas with 11 fungal taxa it was high in the root system of plants from a woodland. Plant species composition and richness was found to basically depend on the diversity of AM fungi (van der Heijden et al. 1998). Eight of eleven plant species on European calcareous grasslands were almost completely dependent on the presence of AM. A study on the genetic diversity of AM on grasslands in Switzerland showed that 10 individual *Glomus* spores from the field produced 10 different genetic patterns, which indicates that the (genetic) diversity of AM fungi in natural communities may be much greater than expected from species determination based on morphology (Sanders et al. 1995). Species richness for higher fungi (as well as for plants and animals) was found to be far greater in more highly stressed plots (environmental disturbance), which were also lower in altitude (Bonavita et al. 1998). This effect may have dominated the results of the study, since a decline in species richness with increasing altitude is widely accepted as general pattern (Rahbek 1995). However, the two data sets of Bonavita et al. (1998) and van der Heijden (1998) can not directly be compared, as the higher fungi assessed in the first study (i.e. ectomycorrhiza and saprophytes) belonged to a different ecological group as those studied by van der Heijden (AM). It is likely that environmental disturbance can positively influence biodiversity in natural environments, while in agricultural environments (the relatively low) biodiversity is affected rather negatively.

Environmental disturbance will affect mycorrhiza (AM and EM) that are obligate biotrophs and depend on the plant partner for carbon supply, more than saprophytic species that have to adapt to changing situations regularly. Interestingly, biodiversity of leaf litter decomposing microfungi was negatively affected by disturbance in a tropical forest, whereas species richness of slime molds and functional diversity of bacteria was increased (Lodge 1997). For the mycorrhiza (AM, EM) it is evident from the data above that the diversity of the fungi can be positively influenced by the diversity of the plants. If this can also be assumed for saprophytes, the diversity of microfungi in soil depends on both plant and animal biodiversity. However, due to differences in ecological functions of AM and saprophytic microfungi, the AM cannot directly be taken as a model for saprophytic fungi.

8.3.2 DIVERSITY OF HYPHOMYCETES: INTERACTIONS WITHIN THE TROPHIC LEVEL

In contrast to arbuscular mycorrhizae, other filamentous fungi have by far not been so extensively studied. In contrast to higher fungi (Basidiomycetes and Ascomycetes with macroscopic fruiting bodies), the microfungi in general, but especially soilborne hyphomycetes, are cosmopolitan species with a worldwide distribution. This is obviously due to their ecological function as saprophytes and the mechanism of airborne dispersal. The wide range of microfungi in soil is saprophytic and thus not directly involved in plant nutrition. But, the saprophytes can influence the growth of microorganisms and crop plants indirectly by renewal of nutrient supply in soil. Moreover, they can influence the growth of both microorganisms and plants by allelopathic effects on the

plants themselves or on the microbial community in soil. The definition of *allelopathy* refers to “any process that involves secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of biological systems” (Anaya 1999). Among the great variety of secondary metabolites found in plants, phenolics and terpenoids represent the main allelopathic agents currently known. Aromatic compounds such as phenols, phenolic acids, cinnamic acid derivatives, coumarins, flavonoids, quinones, and tannins have been identified as allelopathic agents in more cases than all other classes of compounds combined (Inderjit 1996). A wide variety of phenolic compounds, *terpenes*, and terpene derivatives has been found to be produced by microfungi (Larsen and Frisvad 1995a, 1995b; Fischer et al. 1999; Fischer 1994). For each species several compounds were described, but it is extremely complicated to study these effects on natural substrata or *in situ*, as the production widely depends on substrate composition. There are some indications in the literature that the metabolite production of fungi was not strongly affected by other microorganisms tested in combination. Larsen (1996) found in mixed cultures of *Penicillium commune* and *P. roqueforti* metabolites that were known to be unique for either species. Filtenborg and Frisvad (1990) investigated the profiles of secondary metabolites in mixed cultures in view of a direct identification. Not all metabolites of a profile from pure cultures were found, partly due to competition, but a few metabolites turned out to be sufficient for proper identification of the species involved. These results may be taken as an indication that even in natural environments, with all competition operative, species-specific metabolites can be found. A first attempt to characterize the microbial composition in soil by the emission of volatiles was made by Stahl and Parkin (1996), who tried to relate the structure of the microbial community to soil VOC emissions. This connection turned out to be complicated because VOC may be derived from a variety of sources and their production is strongly influenced by environmental conditions. However, the VOC emission from plant material or the VOC production by fungi themselves may be possible factors in fungal competition. Antagonistic effects of volatile microbial metabolites (fungi and bacteria) on other microorganisms and insects have been reported (Barr, 1976; Vanhaelen et al., 1978; Dowd, 1992; Lanciotti and Guerzoni, 1993). An inhibitory effect on *Aspergillus flavus* was found for volatiles of *Rhizopus arrhizus*, which is extremely interesting in view of *A. flavus* being an important mycotoxin producer on food and feed. If single compounds of one species may have significant effects on the growth and reproduction of another species, the complexity of effects possibly occurring in a very diverse fungal community in soil is enormous. Belaiche et al. (1996) studied the antifungal activity of three terpene alcohols, citronellol, menthol, and terpineol, on mycelial elongation of *Aspergillus parasiticus*, *A. flavus*, and *A. niger*. *A. parasiticus* was found to be the most sensitive species and citronellol was found to be the most effective terpene, followed by menthol and terpineol. Moreover, the presence of citronellol increased the antifungal efficiency of both menthol and terpineol. To citronellol all species were similarly sensitive, whereas against menthol and terpineol *A. parasiticus* and *A. flavus* were more sensitive than *A. niger*. Ogawa and Isshiki (1996) showed that volatiles derived from herbs can have antimicrobial activity. Aldehydes such as citral, cinnamaldehyde, and salicylaldehyde turned out to have good antifungal properties. The fungi included in the experiment were *Penicillium islandicum*, *Fusarium oxysporum*, *Aspergillus oryzae*, and *Rhizopus oryzae*. In environments with high concentrations of such volatiles, an antifungal activity may possibly influence species composition. Until now, only limited information on such effects is available. However, the capacity of certain volatiles and nonvolatiles (mycotoxins) to inhibit fungal growth *in situ* may be a factor that influences species composition and diversity in soil in general.

In plant pathology, numerous studies were concerned with the production of toxins or antibiotics *in situ* because of their implication for biological control of plant pathogens. A production of antibiotics in natural environments would increase the competitive ability of a species. Many ecologists believe that antibiotics are active in nature (Wicklow, 1981). Some of the strongest arguments upon which this conclusion is based were summarized already by Brock in 1966:

- Antibiotics can be extracted from nonsterile soil.
- Antibiotic production will occur in nonsterile soil inoculated with antibiotic-producing organisms only when supplemented with organic enrichments.
- Antibiotic-producing microbes can be isolated easily from natural soil.
- Antibiotic-producing organisms are able to antagonize other organisms sensitive to antibiotics.
- Conditions unfavorable for the accumulation of antibiotics reduce the antagonistic activity of the organism producing the antibiotic.

In contrast, microbiologists have argued that antibiosis can only take place on nutrient-rich substrates (Wicklow, 1981). At low levels of available organic carbon, antibiotic synthesis is unlikely to give an advantage to its producer. Specific soil microhabitats in which nutrients are abundant, such as those associated with freshly incorporated organic detritus, have long been recognized as sites where antibiosis is likely to occur (Brian, 1957). Antibiotic production is also probable when a fungus first invades fresh plant tissue, such as in the case of patulin production by *Penicillium expansum* on apple fruits. Consequently, mycotoxin production can be expected to occur in native soil when organic amendments (e.g., fresh biowaste) is incorporated. But the amount of mycotoxins or antibiotics is certainly reduced by metabolic processes during further decomposition of such amendments.

8.3.3 INTERACTIONS WITH OTHER TROPHIC LEVELS

Besides the well-known basidiomycetes cultivated by leaf cutter ants (Weber 1972), other fungi are also associated with ant nests (Folgarait et al. 1996). Anthills may create patches of mycorrhizal enrichment (Fries and Allen 1993), which can help the establishment of plants (Allen et al. 1989) due to the dispersion of fungal spores (McIveen and Cole 1976). Nonmycorrhizal microbes also seem to favor plant productivity in richer anthill soils (McGinley et al. 1994). It can be estimated that apart from a change of the physical properties of soil by construction of nests, also the availability of nutrients may change due to the incorporation of organic material into soil. Soil-inhabiting ants (e.g., *Lasius flavus*) widely feed on honeydew from root-inhabiting plant lice, which they protect against predators. Such habitats may be niches for rapidly developing microfungi more specialized on free sugars, which could then be followed by other species. Similar effects can be observed in air-borne microfungi on plant surfaces that are colonized by aphids. The honeydew on the leaf surfaces, being extremely rich in free-sugars, is rapidly utilized by dematiaceous fungi (e.g., *Cladosporium* species). In how far soil-inhabiting ants in agricultural ecosystems may promote fungal growth in soil and thus benefit plant growth would be an interesting topic to study in detail.

Interesting observations have been made for ectomycorrhiza, which are considered the most important biotic interaction (mutualistic association) in most ecosystems, especially forests. It was suggested that ectomycorrhiza are an important food source for various fungivorous and detritivorous fauna (Sanders and Fitter 1992), and increased nutrient availability in the presence of fungal grazers that had been shown to concomitantly enhance plant growth (Setälä and Huhta, 1991). In spite of a reduction in mycorrhizal biomass by a functionally diverse soil fauna, the N-content and biomass of plants were significantly higher in the presence of fungal grazers (Setälä 1995), obviously due to accelerated nitrogen turnover.

8.3.4 IMPACT OF ANTROPOGENIC ACTIVITY

In the last decades the intensive cropping in agriculture and horticulture was mainly based on the extreme application of pesticides. Pesticides, especially those with broad-spectrum activity, not only control the target pest or disease but may also affect the natural enemies of pests and diseases. Such kinds of pest management lead to an impoverishment of the ecosystem, causing a decrease

in biodiversity and in addition may lead to an enhanced development of pathogens. Moreover, populations of pests and plant pathogens can develop resistance to pesticides, particularly in intensive agriculture where the frequent use of chemicals imposes enormous selection pressure. The development of resistance and the resurgence of pests and diseases required the application of higher doses or more frequent application of chemicals or the introduction of new generations of pesticides. The efficacy of crop rotation as a control measure can be limited when a pathogen has a wide host range and is able to survive long periods with special resting structures, like sclerotia or chlamydospores, in case of fungal pathogens. Moreover, crop rotation is often neglected as it is no longer economic when pesticides can be applied. Apart from the fact that a pathogen can develop resistance against plant-protecting chemicals, the detrimental and toxic effects of those xenobiotics on the environment are of greater concern.

With the increasing awareness of environmental consequences of fungicides and a decrease of available compounds for disease and pest control, alternative biological methods of disease control gain in importance. These biological control agents could either be used alone or in combination with low doses of pesticides as integrated control measures.

Application of the fungicide fenpropimorph (corresponding to the recommended field dose) during decomposition of barley roots lowered the number of total culturable bacteria significantly, whereas culturable *Pseudomonas* and actinomycetes were not affected (Thirup 2001). These findings suggest a possible effect on soil fertility. In the same study the diversity of total bacteria was measured by DGGE and was found to be unaffected by fungicide treatment, which is not surprising as dying or inactivation of microorganisms is not necessarily connected with the destruction of the DNA. Although there are only few results existing on the effects of pesticides on bacterial diversity, the literature shows that saprophytic fungi, protozoa, and some bacteria are harmed, possibly through changed interaction patterns (Thirup 2001).

In general, an increase in species richness can be achieved by amendments of organic materials in soil. Practices such as agroforestry, mulching, legume inoculation, and minimum tillage are applications of metabiotic techniques and can improve soil fertility, whereas ecosystems can also be degraded through human activity (e.g., forest destruction, irrigation with saline water, strip-mining, deep tillage). The latter practices lead to an inevitable reduction of species of animals and plants (Waid 1999).

Setälä et al. (1998) investigated a number of microcosm studies and drew conclusions that are of major importance for the productivity in agricultural ecosystems:

- Trophic level diversity has clear impacts on primary production.
- Declining species diversity alters carbon mineralization in heterotrophic systems due to changing interactions between trophic levels.
- Species composition within a functional group can affect biomass production of plants.

As soilborne hyphomycetes are mostly saprophytes and belong to the same trophic level, trophic level diversity will not be discussed in detail in this chapter. However, metabiotic effects can create niches that may in some way function as different “infra-trophic” levels, for which functional diversity may apply.

8.4 SPECIES RICHNESS AND DIVERSITY IN AGRICULTURAL ENVIRONMENTS

8.4.1 DATA FROM CULTURE-BASED TECHNIQUES

In an investigation in continental Antarctica carried out in soils with algae and cyanobacteria as sole primary producers, microfungi were found to be as abundant as in many fertile cultivated soils in the temperate region (Ugolini and Starkey 1966). Species of *Aspergillus*, *Penicillium*, and

Neurospora were reported. Geothermal areas with mosses and liverworts had a greater variety of fungi (Broady et al. 1987), with *Chaetomium*, *Malbranchea*, *Mucor*, *Myceliophthora*, and *Paecilomyces* also occurring. As in such surveys certain taxa are not completely identified to the species level, their relevance for comparison with respect to biodiversity is limited.

Investigations on population density of filamentous fungi in Maritime and Subantarctica showed that the density of microfungal hyphae in soil is correlated with vegetation type and abundance, overlapping the abundance in a somewhat similar temperate soil (Heal et al. 1967). Mineral soils contained 4–147 m hyphae per gram soil (dry weight), grassland 288 m, and moss peats 2783–6328 m (Bailey and Wyn-Williams 1982).

The ratio of fungal to bacterial biomass (measured by phospholipid fatty acid analysis, or PLFA) was consistently and significantly higher in the unfertilized than the fertilized grassland (Bardgett and McAlister 1999). Although there was evidence that the microbial biomass (measured as chloroform fumigation and total PLFA) was higher in the unfertilized grassland, it was found that levels of inorganic nitrogen were significantly higher in the fertilized grassland.

My own investigations in view of characterizing the spectrum of soil fungi on flower bulbs in relation to *Rhizoctonia* disease showed that species richness was affected by physicochemical properties of the soil, but was not correlated with disease suppressiveness (Fischer, 1994). During the whole investigation 63 species were isolated, some of which showed slight preferences for a certain location. However, all these species did not markedly differ in abundance between the three location studies and, consequently, none of these fungi can be made responsible for differences in suppressiveness of the soils. The somewhat higher species diversity in one of the three locations was correlated with some physicochemical characteristics of the soil. This soil had the highest content of organic matter and the highest water-holding capacity. This highly loamy sand was less sensitive to desiccation during the summer, which otherwise may have influenced diversity. A high content of organic matter, the high loam content, and the low C/N ratio at this location were favorable factors for microbial multiplication. This may have lead to a higher probability of isolation for rare species, what finally becomes apparent in higher species numbers (diversity). Greenhouse experiments in a phytotron with soil samples from the three locations yielded 24, 9, and 5 additional species that had not been found during the field isolation. This indicates that the number of samples taken from the field was not sufficient to characterize the fungal community.

8.4.2 DATA FROM MOLECULAR METHODS

Apart from culture techniques and direct microscopy, molecular methods have been used to characterise microbial communities in soil (e.g., denaturing gradient gel electrophoresis, or DGGE). Kowalchuk et al. (2003) described changes that soil treatments (i.e., fumigation, flooding, sterilization,

TABLE 8.1
Species–Frequency Relation (data obtained with SEA and
RMPA in all experiments combined)

Number of recordings	Number of species	Percentage of species
1	19	30.2%
2–5	14	22.2%
6–10	8	12.7%
11–20	9	14.3%
21–30	2	3.2%
31–40	2	3.2%
> 40	9	14.3%
Sum of species	63	100.0%

compost amendment in comparison with untreated soil) induced in the microbial community in order to determine if particular microbial components of the system could be associated with suppression of soil against *Phytophthora* root rot. One result was that the compost treatment of sterilized soil appeared to have the most profound effect on the dominant microbial populations. The application of DNA-based molecular techniques is very questionable in this case, as the DNA of the microorganisms is present in the soil even after the treatments applied. Although microorganisms might be inactivated by sterilization, DNA can only be effectively destroyed by UV-irradiation. The spectrum of microorganisms changed when compost was amended, because additional taxa were introduced into the substratum. Another result was that the suppressive activity against *Phytophthora* in compost-amended soil was associated with a different microbial community than the one observed in untreated, suppressive soil (see also van Os and van Ginkel 2001). In this case the results seem to be more reliable and are of significance for disease management in horticulture. The spectrum of fungi identified by the DNA fragments obtained from DGGE was very sparse, resulting in two species of *Chaetomium*, *Geomyces pannorum*, *Acremonium* sp., *Microdochium bolleyi*, and some yeasts (i.e., *Cluyveromyces* sp., *Candida fermenticaria*, and *Ascotricha chartarum*). The species richness (10 species using two primers) did by no means reflect the diversity of microfungi to be expected in compost or soil. Thus, such approaches are rather inadequate to characterize both species and/or functional diversity.

Another method for the assessment of the fungal diversity and identification of fungi is the oligonucleotide fingerprinting of rRNA genes (OFRG) (Valinsky et al. 2002a). OFRG sorts arrayed rRNA (ribosomal DNA) clones into taxonomic clusters through a series of hybridization experiments, each using a single oligonucleotide probe. A simulated annealing algorithm was used to design an OFRG probe set for fungal rDNA (Borneman et al. 2001). Valinsky et al. (2002b) used this method to characterize the fungal diversity in field soil to identify the microorganisms involved in soil suppressiveness against the plant-parasitic nematode *Heterodera schachtii*. Using the above array the analysis of 1,536 fungal rDNA clones generated 455 clusters (criterion was 99.2% similarity). (Identification results were cross-checked with nucleotide sequence analysis of 117 clones.) Eighty percent of the clones belonged to the *Ascomycota*, 12% belonged to the *Basidiomycota*, with a large fraction of *Fusarium* (404 clones) and *Raciborskiomyces* (176 clones). Furthermore, smaller groups showed sequence identities with *Alternaria*, *Ascobolus*, *Chaetomium*, *Cryptococcus*, and *Rhizoctonia*. All in all, such a method can handle a large number of clones, but the number of taxa assessed on the species level is rather low compared to culture-based techniques.

Microbial communities differing in biodiversity were established by inoculating sterile agricultural soil (clay loam soil, crop: *Phaseolus vulgaris*) with serially diluted soil suspensions prepared from the parental soil. Biodiversity decreased by each successive dilution step, but no consistent effect of biodiversity on a range of soil processes could be observed (Griffiths et al. 2001) (i.e., nitrate accumulation, incorporation of amino acids, potential nitrification, respiratory growth response, community level physiological profile, and decomposition). Astonishingly, the experimental reductions in biodiversity had no direct effects on soil function.

Direct extraction of DNA (18S rDNA) from soil followed by PCR and separation of amplicons via DGGE did not show differences between different sampling sites and farming management systems (i.e., precision and conventional farming), and only small differences were observed throughout the vegetation period (Hagn et al. 2003a, 2003b). Contrarily, with the cultivation-based method clear site-specific and seasonal effects were observed for the fungal community structure. The authors concluded that the molecular approach (cultivation independent) is not influenced by the various factors investigated, whereas active populations show a clear response to environmental changes. These results clearly demonstrate that classical cultivation-based techniques are still an important prerequisite to characterize functional diversity of fungi in soil. Molecular approaches can help to detect microorganisms that are under certain conditions not culturable, may be outnumbered by abundant species, or are present in an inactive form (resting stage) in soil.

It is interesting to realize that none of the authors cited above even discussed how far the results could be influenced by free DNA from dead organisms in soil. It is necessary to know which microorganisms grow actively in soil and which ones are in some kind of resting stage and might be active in time. The amplification of target DNA (e.g., rRNA genes) from bulk samples can bear the risk that “waste” DNA without any implication for the abundance and activity of fungi in soil is detected. What is the significance of results that show that the spectrum of DNA amplicons is (1) identical before and after sterilization of soil samples or (2) identical throughout different seasons?

8.4.3 COMPARISON OF BIODIVERSITY ASSESSED BY CULTURE-BASED AND MOLECULAR TECHNIQUES

Moreover, it got obvious that the number of taxa assessed with molecular techniques is rather low compared to classical cultivation-based studies. To study biodiversity adequately, much more stress must be laid on the accurate identification of microfungi by classical methods of cultivation and microscopy. The strong need for a taxonomic mycologist was colorfully described by Hyde (1996), who mentioned that 32 mycologists are working on up to 250,000 species of fungi, while 25 are working on 600 species of *Eucalyptus* sp.

8.5 SPECIES RICHNESS AND DIVERSITY IN NONAGRICULTURAL ENVIRONMENTS

A study of the soil microfungal community at the western shore of the Dead Sea resulted in 78 species from 40 genera (70 samples taken) (Grishkan et al. 2003). The substratum examined was sand, which can be characterized as extremely stressful due to xeric and oligotrophic conditions. Melanin-containing micromycetes (46 species, 65.5%) were the dominant group, followed by teleomorphic ascomycetes and soil and plant surface-inhabiting species. The regularly occurring species were considered the characteristic micromycete complex for the coastal habitat (i.e., *Alternaria alternata*, *A. raphani*, *Aspergillus niger*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Ch. murorum*, *Cladosporium cladosporioides*, *Penicillium aurantiogriseum*, and *Stachybotrys chartarum*). According to the authors of the study, this confirms the conclusion that there is not a specific halo- or thermophilous mycobiota characteristic for these habitats. It is likely that the dematiaceous species (e.g., *Alternaria* and *Cladosporium*) are rather airborne and enter the substratum regularly via air (samples were taken from the upper 1–3 cm of the substratum). These species are resistant to drought and UV radiation due to the melanin. The *Chaetomium* species may be airborne but can as well colonize little wood particles. The abundance of *Stachybotrys* sp. is rather interesting, as this species is also characteristic on wooden substrates, but is not likely to enter the substrate in significant numbers by air. A problem that has been addressed above as the “stability” of nomenclature (Vishniac 1996) gets obvious in the work by Grishkan et al. (2003). *Penicillium aurantio-griseum* is regarded as a species complex for some years. It does not get obvious from the references cited if this new system has been applied and, thus, information is not available on the reliability of the identification of this species.

Diversity of soil microfungi was surprisingly high on abandoned industrial deposit areas with elevated levels of metals (Mn, Zn) in the Czech Republic (Kubatova et al. 2002). At an ore-washery settling pit a number of 94 species were found, at an ash-slag settling pit 46 taxa were isolated. Taking other ecological groups into account (e.g., root associated, coprophilous, parasitic microfungi, AM, macrofungi, and lichens), altogether 219 species were found in the first location, but only 72 in the latter due to a lower number of samples investigated and an earlier stage of succession. The dominant soil fungi were said to differ from those of natural soils; the most frequently isolated species were *Penicillium janthinellum*, *P. simplicissimum*, *Cunninghamella elegans*, *Paecilomyces lilacinus*, *Trichoderma virens* and *Trichoderma* spp., *Mucor hiemalis* f. *hiemalis*, *Coniothyrium*

TABLE 8.2
Species Richness and Biodiversity of Fungi in Different Agricultural Environments

Object/substratum studied	Method	Number of isolates	Number of genera identified	Number of species identified	Number of clones	Authors
Field soil, iris bulbs, The Netherlands	Cultivation based, plating of bulb/root fragments, washing of fragments	340	36	63	—	Fischer 1994
Field soil, corn-soybean rotation, low-input system	Cultivation based, dilution plating	18,000	40 e.g., <i>Penicillium</i> <i>Myrothecium</i> <i>Fusarium</i>	n.a.	—	Buyer and Kaufman 1996
Field soil, Triticum aestivum cv. Baldus	PCR, temperature gradient gel electrophoresis (TGGE)	n.a.	22	24	61	Smit et al. 1999
Suppressiveness against <i>Heterodera schachtii</i> , field soil	Oligonucleotide fingerprinting of rRNA genes	n.a.	7	n.a.	1,536	Valinsky et al. 2002a, 2002b
Field soil, winter wheat	Cultivation based, soil-washing/parallel to DGGE	866	3 <i>Trichoderma</i> <i>Fusarium</i> <i>Verticillium</i>	4 (<i>Trichoderma</i> types)	—	Hagn et al. 2003a, 2003b
Field soil/compost amended, flower bulbs	DGGE	n.a.	7	10 <i>Chaetomium</i> , <i>Geomyces pannorum</i> , <i>Acremonium</i> sp., <i>Microdochium bolleyi</i>	—	Kowalchuk et al. 2003

n.a. = information not available

fuckelii, *Mortierella alpina*, *Fusarium* spp., and *Coemansia aciculifera*. The stress factors considered to be responsible for the selection of the fungal spectrum were said to be lack of basic nutrients, high metal concentrations, fluctuation of soil temperature and pH, acidification, and secondary salinization. Additional studies focused on the changes in fungal biodiversity during microbial succession in polluted soils. Factors investigated were the level of heavy metal contamination (Nordgren et al. 1985), pollution by alkaline deposition in a coniferous forest (Fritze and Bååth 1993), and succession in a postindustrial soil (Kowalik 1995).

Establishment of for instance plant species not native to a habitat can change the microbial community in the habitat, which was shown for aquatic saprophytic hyphomycetes. Species richness and evenness, but not numbers of waterborne conidia of aquatic hyphomycetes, were significantly lower in Eucalyt-bordered streams. But despite differences in the dominant decomposer community, decay rates of Eucalypt leaves (neophyte, *Eucalyptus globulus*) and Chestnut leaves (natural vegetation) did not differ (Bärlocher and Graca 2002). These results can be taken as an indication that a decrease in biodiversity of saprophytic fungi must not necessarily lead to measurable effects of soil processes such as the time of decomposition. On the other hand, species reduced in abundance or not occurring any more may be ecologically connected to other organisms, that depend on their resource supply and are hence indirectly influenced, for example, in metabiosis (Waid 1999).

8.6 CONCLUSIONS

Metabiotic interactions continuously create new microbial niches that are quickly filled from the resident pool of rare and “cryptic” and also cosmopolitan species (Waid 1999). Finley et al. (1997) suggested that “microbial diversity has no discrete role to play with respect to ecosystem function. Reciprocal interactions between microbial activity and the physical-chemical environment create a continuous turnover of microbial niches that are always filled, so microbial diversity is a part of ecosystem function.”

Some studies both in agricultural and nonagricultural ecosystems have indicated that a decrease in biodiversity of saprophytic fungi must not necessarily lead to measurable effects in soil processes such as nitrification, respiratory growth response, and decomposition (Griffiths et al. 2001; Bärlocher and Graca 2002). Astonishingly, it seems that under some circumstances a reduction in biodiversity does not have a direct effect on soil function, which means that productivity in agricultural ecosystems may be unaffected at first. But a reduction in biodiversity is likely to get visible whenever pathogens can develop abundantly and lead to serious problems in crop production. Therefore, also with respect to soil fungi, biodiversity seems to be crucial for ecosystem stability.

Finally, both the number and partly the quality of investigations carried out to assess species richness (taxonomic biodiversity) and genetic biodiversity is low and a lot more research is needed to clarify the role of soil fungi for ecosystem stability in agricultural environments. Methods to describe the diversity of the genetic resources (TGGE, DGGE, OFRG) in soil fungi have been applied scarcely but the experimental setup and the performance of studies with soil fungi need to be significantly improved. A combination of molecular and culture-based, in combination with direct, microscopy techniques need to be applied to reliably assess data on biodiversity (genetic, taxonomic, and functional biodiversity). The physical environment is consistently changed by the variety of agricultural techniques and data are currently very sparse to describe changes in biodiversity due to the physical environment. Human management practices have changed from intensive to extensive methods during the last few years. There are strong indications that amendment of soil with complex organic substrates such as compost can significantly alter the biodiversity of agricultural soils due to introduction of a diverse spectrum of microfungi.

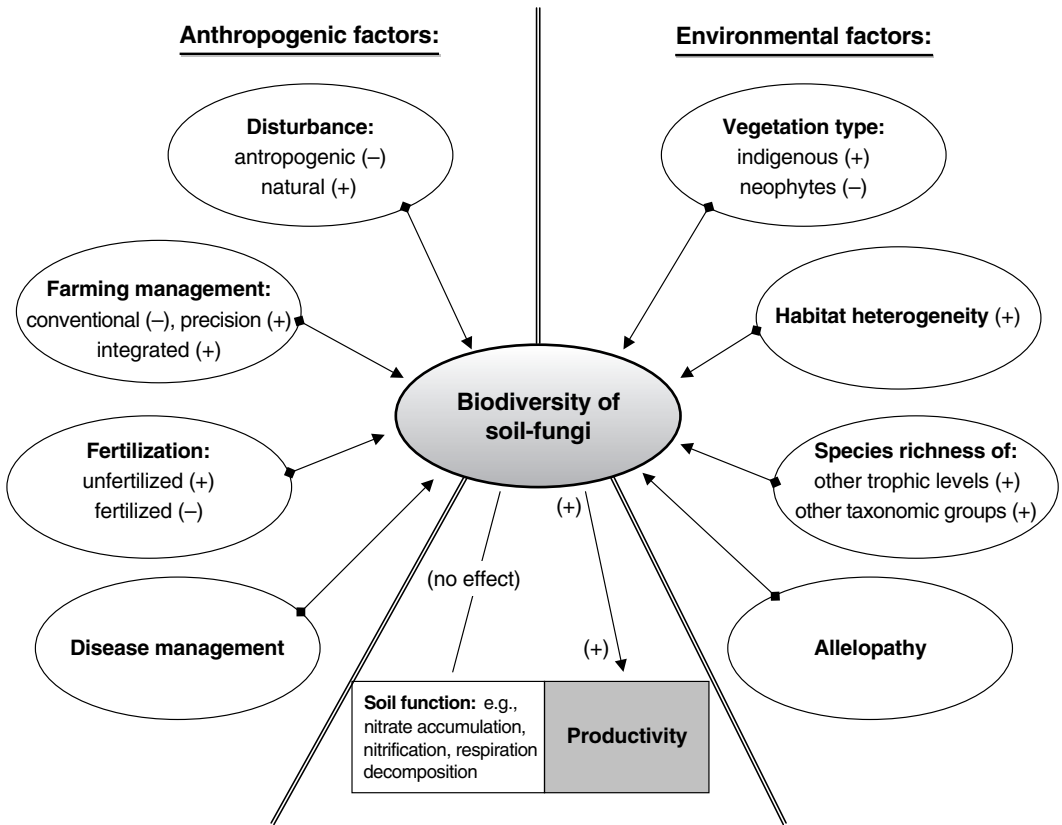


FIGURE 8.1 Factors influencing biodiversity positively (+) or negatively (-) and the impact of biodiversity on soil function and productivity.

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9 Diversity of Chytrids

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9.1 WHAT ARE CHYTRIDS?

The Chytridiomycota (chytrids) are fungi that have a motile stage involving posteriorly uniflagellate (in most cases) zoospores.^{1,2} These organisms are widespread in terrestrial and aquatic ecosystems where many are saprophytes, especially utilizing polymeric organic substrates. Others are microscopic parasites of various soil- and water-inhabiting organisms (algae, rotifers, nematodes, tardigrades, other fungi, including chytrids) as well as plants and amphibians. In terms of size, in most cases chytrids do not form visible growths. Dayal³ has noted that these organisms “rarely develop in sufficient abundance to be recognized directly in the field” (p. 292). The chytrids are basal to the true fungi. Evidence of an *Allomyces*-like organism has been found in the Devonian Rhynie Chert,⁴ and the nonzoosporic fungi appear to have separated from the chytrids approximately 550 million years ago, as discussed by Berbee and Taylor.⁵ The fungi are grouped within the opisthokonts, (Baldauf^{6,7}), a clade that includes the animals, choanoflagellates, and microsporidians.

The chytrids have been studied extensively. By the 1830s chytridiaceous fungal taxa had been established, and careful sketches soon were available. A frequently cited early drawing by A. Braun, from 1856, shows *Chytridium olla* parasitizing the oogonium of the alga *Oedogonium* (fig. 9.1). In this rendering, discussed by Sparrow,⁸ two chytrids (a chytridion or “pot” or “container” in Greek, and thus the genus *Chytridium* and the word “chytrid”) are attached to the algal oogonium surface. Within the alga, broad, unbranched, penetrating haustorial structures and a resting spore also are

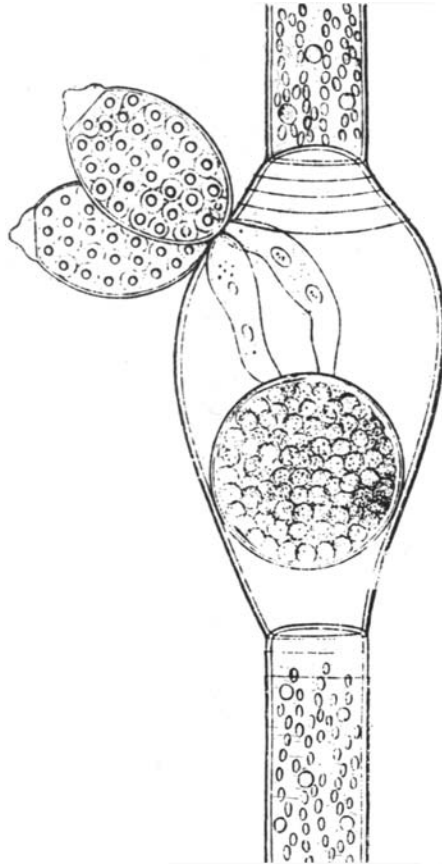


FIGURE 9.1 A drawing by A. Braun, completed in 1856, of the chytrid *Chytridium olla* parasitizing an alga. Two pot-like (thus described as a “chytrid”) thalli, on the surface of the algal Oogonium, broad haustorial tubes within the alga, and an endogenous resting spore, possibly associated with this chytrid, are evident. Reprinted from Sparrow.⁸

depicted. Eventually, motile zoospores will be released from the chytrid thallus (body) that will colonize new substrates and repeat the cycle.

The basic life cycle of a widely studied chytrid, *Synchytrium brownii*, is shown in figure 9.2. This chytrid, that does not develop a rhizoid structure, reproduces using either an asexual or a sexual cycle. In both cases, the motile zoosporic phase attaches to and penetrates the host (in this case a plant), and the thallus will be formed. The sexual cycle involves plasmogamy, karyogamy, and resting spore formation. Triggered by environmental clues, germination and meiosis may occur, and after the formation of zoosporangia, haploid zoospores can be released. Other chytrids exhibit variations on this theme, however many species carry out (as far as is known) only the asexual portion of the cycle. In some organisms, the body (thallus) of the organism only attaches to the surface; in others it actually penetrates the living host or nonliving substrate.

With such variations in life forms, only considering these limited examples, the chytrids present many challenges for assessing biodiversity. As discussed by Barr,⁹ chytrids can be (1) *monocentric* or *polycentric* (having a single or multiple reproductive structures), (2) for monocentric species, being *holocarpic* (the entire organism is involved in reproduction, the thallus does not have rhizoids) or *eucarpic* (having a thallus plus nonsexual rhizoid structures), and (3) showing differences in nuclear movement. If the nucleus remains in the thallus, it is described as undergoing *endogenous* development, or if it moves into the germ tube or rhizoid during the growth process, as *exogenous*

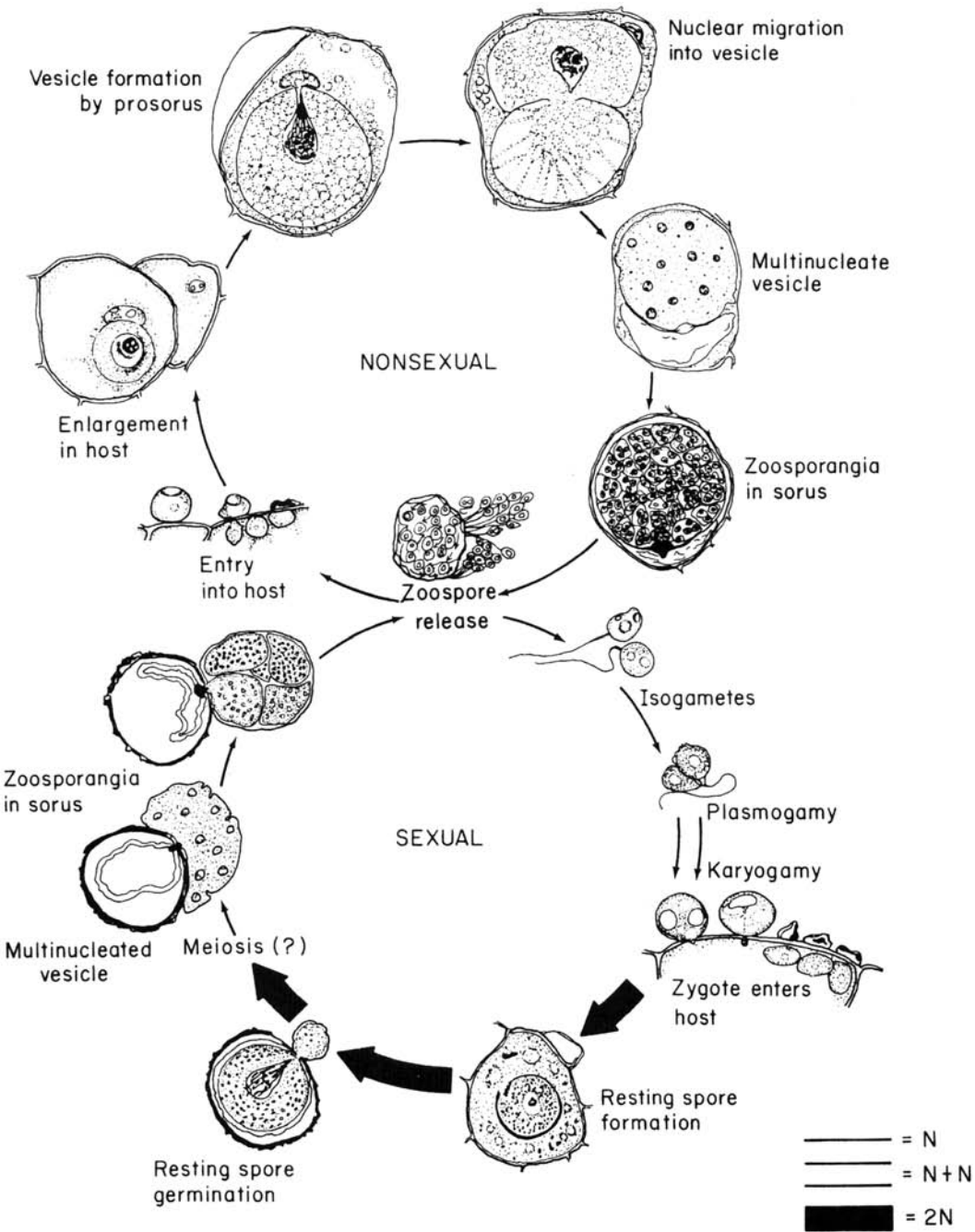


FIGURE 9.2 The life cycle of *Synchytrium brownii*, including nonsexual and asexual cycles. Reprinted from Moore-Landecker.²

development. These variations in physical characteristics lead to five types of basic thallic structures: (1) endogenous–monocentric, (2) endogenous–polycentric, (3) exogenous–monocentric, (4) colonial (exogenous–polycentric), and (5) filamentous (exogenous–polycentric). These structures range from the simplest to the most complex and are useful in classification and identification of taxa at the order level,¹⁰ as well as for describing more general aspects of diversity.

It is important to emphasize that the term “chytrid” can be used in different ways, and that its meaning has changed in the recent past. Earlier, this was used only to describe the members of the orders Chytridiales and Spizellomyces before Barr¹¹ separated these orders. Barr also suggested that the term be reserved for the Chytridiales, but acceded to the prevailing use of the word to mean a member of the Chytridiomycota.⁹

Of special importance for attempting to understand the biodiversity of zoosporic fungi, as discussed by Sparrow,¹² are the careful studies of Scherffel.^{13,14} In his work, he examined small twigs recovered from Lake Balaton, Hungary, including some that were preserved in alcohol by a colleague. He described new species, including *Rhizophydium clinopus* and *Chytridium pusillum*, that are of special interest. He noted the ability of the zoosporic swimmers to select or reject potential host cells. Penetration of the host cells involved contact by the leading side of the zoospore, and multiple infection frequently occurred. This resulted in the formation of “midget” zoosporangia, only contained two to three zoospores. He used the “Lagerheim method” which, as he noted, increased the ability to discover and describe these organisms. The method of Lagerheim,¹⁵ used in his studies, involved the direct examination of plant materials without the use of baits. The essence of the method involved transferring twigs to clean water, where they were kept at the water surface, emphasizing the importance of maintaining aerobic conditions for these zoosporic fungi. In addition, it was important to avoid excessive organic matter additions to minimize overgrowth of other organisms, primarily bacteria. He also noted that the members of the Monoblepharidales were especially attracted to areas on the twigs where the bark had fallen away, leaving the cortex of the twig exposed.

Lagerheim also described the interactions of zoospores and oogonia, and the role of attractants, assumed to be chemicals released by the oogonia, that led to changes in zoospore motility and direction of movement, followed by contact and eventual penetration of the oogonium by the zoospore. In more recent studies, Powell¹⁶ also has noted the ability of zoospores to recognize chemical clues and to select suitable hosts, indicating a high degree of host specificity. *Catenaria* is a particularly interesting example; this is attracted by chemical clues to colonize nematodes. For *Allomyces*, the pheromones sirenin and parisin play important roles in taxis phenomena and reproduction.

The zoospore, as discussed by Carlisle,¹⁷ has as its sole purpose “the transport of the fungus genome from one suitable environment to another.” Motility requires energy and this is provided in the form of lipid droplets in many species. In some zoosporic fungi glycogen is used as an energy storage reserve, and others, such as *Blastocladia*, are fermenters. These motile forms that move in water films search for new niches to exploit. This involves selection of a specific host or substrate, instead of simply depending on the indiscriminant mass production and release of sexual and asexual spores, as occurs with many of the filamentous fungi. As discussed by Letcher and Powell,^{11,18} the zoospore confronts specific spatial challenges as it seeks new substrates and hosts. After release, the unvalled zoospores radiate out through available capillary-bound water films. In flooded soils, zoospores can be moved beyond the localized regions where they were released from the sporangium. A special case is *Septosperma rhizophydii*, which has a rocket-shaped, walled resting spore that can be moved passively with runoff water.¹⁹

The unique lifestyle and the difficulty in observing and studying these organisms in nature, unfortunately, has led to their conceptual and experimental marginalization, particularly their limited inclusion in estimates of microbial biodiversity; the work is very labor intensive, as discussed by Zak and Rabatin.²⁰ With these challenges, very few scientists, including mycologists, specialize in their study, and unless one is a careful observationist, these interesting organisms simply will be missed. They have been considered as having “little importance for soil ecosystems,”²¹ “being of little economic interest,” and as “having little direct effect on human welfare,” comments in the literature noted by Powell.¹⁶ As discussed by Paquin et al.,²² the less well-known lower fungi, including the Chytridiomycota and the Zygomycota, “have been relatively neglected.”

However, for agricultural producers who are directly affected by these organisms, in contrast, these are of major concern, as with potato infestations with black wart disease, an important disease caused by *Synchytrium endobioticum*¹⁶ and brown spot of corn, caused by *Physoderma maydis*.⁹ In addition, other chytrids can serve as vectors for viral diseases of several specialized plant crops²³ while several genera are parasites of amphibians.^{24,25}

9.2 ASSESSING BIODIVERSITY

9.2.1 GENERAL APPLICATIONS IN THE MICROBIAL WORLD

Biodiversity developed as a concept in the world of macroorganisms, and can be expressed analytically in terms of (1) variety indices, (2) evenness indices, or (3) as a Shannon index of general diversity, as classical approaches used to analyzing the structure of populations.²⁶ A critical aspect of such studies is the ability to document the relationship between the number of species (S) and the number of individuals per species (N/S) in a particular community. As noted by Odum, species diversity has a number of components “which may respond differently to geographic, developmental or physical factors.” A fundamental problem, which affects work on microbial diversity, including work with the chytrids, is that in macroorganisms these considerations are based on the individual, whereas with microorganisms the major emphasis is on populations.²⁷ In spite of this fundamental difference, concepts of diversity, developed in the world of macroorganisms, are often applied directly to microorganisms with few caveats. As discussed by Gest,²⁸ use of the word *diversity/biodiversity* in the world of microorganisms can be based on one of several meanings, and that “the one in mind is frequently not specified.” Aspects of this problem, as discussed by Gest, include the assumption of the suitability of molecular approaches in providing information on biodiversity without the need to cultivate microorganisms, and the “myth of unculturability,” or the widely expressed assumption that most microorganisms that are observed in nature cannot be grown.

Much depends on the methodology that is being chosen and what specifically is being counted. For biodiversity assessments, microbial populations (including the chytrids and other zoosporic organisms) can be assessed based on: (1) direct observation in the environment without considering possible activity; (2) direct observation, with assessment of active versus inactive forms, based on *in situ* approaches; (3) growth-based assessment, with responses assessed using modified *ex situ* cultural conditions (culture media, etc.), or modified *in situ* conditions, by addition of desired “baits” or growth substrates; (4) the use of indirect (molecular/phylogenetic) approaches, where nucleic acids are extracted using bulk recovery procedures, followed by cloning and sequencing; or (5) the recovery of individual microbes determined to be functioning as active members of the microbial community, followed by their physiological and/or molecular assessment as individual isolated organisms.

Each of these approaches provides different information on the biodiversity of the microbial community. Fungi that are included in a particular index of “biodiversity” may be present in the environment, but not be part of a functioning microbial community. As a particularly important example, fungi of various types can be derived from resting propagules that may have been recently deposited in the particular environment through dust, precipitation, or transport by insects, animals, and birds, etc. This problem of being present in a given locale versus being part of a functioning microbial community is of long-standing concern; Waksman²⁹ noted that “the question is not how many numbers and types of fungi can be found in the soil, but what organisms lead an active life in the soil.” In addition, he discussed the problem of spores being brought in “by some outside agency.”

To emphasize this concern, Harley and Waid³⁰ stated that “one of the first requirements for an ecological study of soil organisms is that the methods must distinguish between organisms which are vegetatively active in the soil and play a part in the soil processes and those which exist in dormant or inactive form as spores and other propagules.” Warcup^{31,32} provided similar thoughts. Griffin³³ also has recognized this need: “objectively, the length of viable or better of actively growing

hyphae of each species, along with the frequency of spores, are necessary to quantify the fungal population.” An even more succinct expression of these concerns has been provided by Dick³⁴: “The mere isolation of a species from a particular site provides no evidence of its origin or potential activity in relation to that site.” This problem also has broader implications; individual organisms may be inactive when observed or sampled; however, the organisms may become functional as environmental conditions (pH, light, moisture, temperature, etc.) might change. This is important as such inactive forms may be integral and necessary players in the community, being expressed only under environmental conditions different from those that existed when the environment was sampled. Sample size also can be important in assessing biodiversity. Frequently occurring organisms are usually recovered regardless of sample size, while with organisms present at lower relative abundance, biodiversity is undervalued with smaller sample sizes, as well as with less intense sampling and less frequent sample observation.

As an additional aspect of this problem, Paulus et al.³⁵ noted that direct and indirect approaches to microfungal isolation are important in determining the value of the derived information; “inactive fungal propagules on the surface of leaf litter may provide an exaggerated and erroneous impression of the diversity of fungal assessment with leaf decay.”

All of these statements apply equally to the assessment of chytrid biodiversity in agricultural production systems. This may be less of a problem with chytrids or other zoosporic fungal-like organisms, as their thalli or resting spores usually are associated with immobile substrates, or with plant and animal hosts. The possibility of movement by other agents (wind/water movement, etc.), however, makes it important to ensure that organisms that are enumerated and considered to be part of a community are actually functioning in the environment.

Biodiversity assessment, based on these considerations, requires that forms active in the environment be observed, recovered, and characterized. Baiting techniques, used in many studies of chytrids,^{18,36–38} do not provide information on the substrates that are actually being used by the chytrids that are observed. Baiting approaches also can lead to enrichment of specific chytrid groups, capable of responding to the particular bait, possibly skewing biodiversity assessments. An exception to this generalization might be the use of baiting as a part of a most-probable-number (MPN) approach to assess chytrid dynamics and responses in perturbation experiments, as carried out by Canter and Lund³⁹ and Lozupone and Klein,⁴⁰ to provide estimates of population end points.

Molecular technique-based assessment of biodiversity, based on bulk DNA extraction from environmental samples, has gained wide acceptance in recent years.⁴¹ The ease of use of these growth-independent approaches to assessing biodiversity, and the caché of applying “cutting-edge” molecular techniques, has led to the assumption by many researchers, even if implicit, that there is no need to grow or understand the physiology of microorganisms to be able to understand and document biodiversity, as discussed by Gest.²⁸ A critical problem is that the source of molecular sequences recovered from an environment often is not known; if they are derived from organisms, and if from organisms, whether they are active in the particular environment.

9.2.2 DIVERSITY AND THE SPECIES CONCEPT IN THE CHYTRIDS

Beyond the obvious differences that may be observable using gross microscopy, species determination among the chytrids remains a formidable task. As noted by Barr,⁹ the major problems include “their small size, general lack of distinctive characters and the morphological variation of any one species when grown under different conditions.” As an example, Sagan and Margulis⁴² noted that fertilized cells of *Blastocladiella emersonii* could develop into three different adult forms, a colorless structure, a dark feeding structure, or a small body that releases a single motile cell. This depended on the particular moisture, nutrient, or carbon dioxide status of the soil environment. The morphology of the thallus (its reproductive and nonreproductive structure) also can vary, depending on environmental conditions.⁹ A major factor influencing morphological assessment is whether natural mixed water culture preparations (with commensalistic bacteria) are examined, or if one chooses to work with laboratory species in pure

culture. Major differences in morphology and physical characteristics can occur when working in these different situations and ideally, the organisms should be described based on both types of cultures. A critical point in all of these studies, as noted earlier by Whiffen,⁴³ is that the chytrids used in many of these studies were not bacteria free. In her work, she found that the characteristics of chytrids varied, depending on whether they were or were not contaminated by commensalistic bacteria. With a series of isolates, Whiffen was able to establish pure cultures for the more specific evaluation of chytrid physiological and morphological characteristics. Some of the chytrids were able to degrade cellulose without the assistance of commensal cellulose-degrading bacteria, while with others, cellulose degradation only occurred if the commensalistic bacteria were present. This provides an additional level of complexity when considering functional biodiversity of these interesting chytrids.

This is also of concern for the zoospore. As emphasized by Barr,⁴⁴ “the size of the chytridiomycete zoospore is variable and affected by the medium.” Shape is less variable, but it may be influenced by zoospore activity; this tends to be useful as a diagnostic criterion at the order level, while within the Chytridiales, it is clade specific. To supplement information on such gross morphological characteristics, ultrastructural characterization of zoospores has proven useful. As discussed by Barr,⁹ flagellar ultrastructure shows clear differences between the different Chytridiomycota orders (fig. 9.3). The value of these ultrastructural data also have been supported by molecular information.⁴⁵

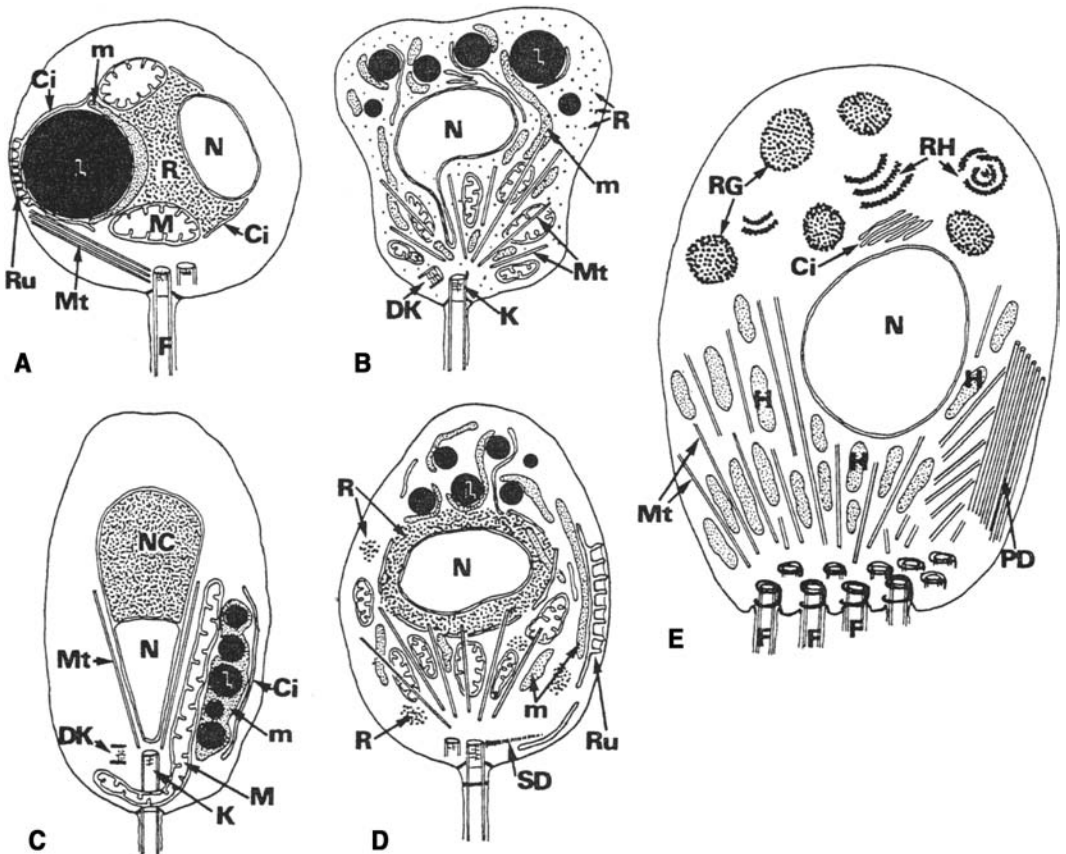


FIGURE 9.3 Zoospore types found in the five orders of the Chytridiomycota, including Chytridiales (A), Spizellomycetales, (B), Blastocladales (C), Monoblepharidales (D), and Neocallimastigales (E). Figure notations: *Ci* Cisterna; *DK* dormant kinetosome; *F* flagellum; *H* hydrogenosome; *K* kinetosome; *M* mitochondrion, *m* microbody; *Mt* root microtubules, parts of the flagellar apparatus; *N* nucleus; *NC* nuclear cap of aggregated ribosomes; *PD* posterior dome; *R* ribosomes; *RG* globules of ribosome aggregates; *RH* helices of ribosome aggregates; *Ru* rumposome; *SD* striated disc. Reprinted from Barr.⁹

Many of the Chytridiomycota are asexual.⁴⁶ Being asexual, “any non-lethal mutation to the genome causing morphological or physiological variation in effect results in the creation of a new species or form,” and “the numbers of species may be infinite.”⁹ In addition, Barr¹⁰ has noted that there is “an indeterminate number of monocentric species in the Chytridiales and Spizellomycetales. Classical classification is untenable.”

Thus, it is critical to apply molecular approaches to begin to solve the problem of chytrid speciation. Without this, there is little hope of reconciling older morphological data with current information on diversity. The recognition that morphological/structural features and molecular approaches to assessment largely provide complementary and confirmatory information⁴⁵ should provide a substantial foundation for further work in this area.

9.2.3 Methodological Aspects of Diversity Assessment

The methods used for chytrid isolation, culturing and identification, have been of continuing concern,^{10,47} especially with the difficulty involved with examination of reproductive and resting structures associated with (often inside of) a host organism or substrate. As discussed by Garrett,⁴⁸ “improved techniques are required for studying colonization of natural substrates by soil fungi.”

9.2.3.1 Statistical and Sampling Concerns

Letcher and Powell¹⁸ documented the effect of even minor changes in environmental characteristics on zoospore fungal occurrence in moss-covered and exposed forest soils. In their study, soils from microhabitats in moss-covered and exposed forest soils were examined in terms of physical scale differences. Organism distribution and frequency were assessed using three baits (sterile pollen grains, split sterile hemp seeds, or boiled shrimp exoskeleton) and mycographs of common zoospore fungi were developed. A major concern, based on their study, is the need to consider scale and sampling in assessment of chytrids in complex forest soils and by implication, in any studies in agricultural production systems.

9.2.3.2 Direct Microscopic Assessment

In terms of direct assessment, the most effective (and laborious) approach to assessing zoospore fungal diversity is direct microscopy. This approach, discussed by Sparrow,^{12,49} involves the immediate examination of large numbers of samples, and large sample sizes. As noted, this is a laborious process, but necessary to be able to observe fungi that are sensitive to environmental changes. This approach was also emphasized by Lagerheim.¹⁵ A related approach that can be linked to direct examination is particle filtration, used to develop relationships between the total number of isolates examined and the number of new isolates that are observed. Rossmann⁵⁰ has noted that this approach will tend to favor the recovery of fungi that are active “in the substrate.” The rarefaction curves that are derived by this approach⁵¹ describe the extent to which organisms have been recovered from the substrate (fig. 9.4); in these cases, the rarefaction curves indicated that the maximum number of species still had not been recovered. Paulus et al.³⁵ also used this approach to determine when maximum recovery of organisms from a given colonized substrate had been achieved. Although some success may be able to be achieved in examining such materials for chytrids using this approach, there are special problems, as the chytrid thallus may or may not be able to be separated from the host structures.

Another useful approach involves the stimulation of zoospore release by flooding soil or mud samples with clean water, to provide a low-nutrient, aerobic environment. Sparrow⁵² discussed the use of this approach, by L. G. Willoughby, to evaluate temporal aspects of zoospore release. With lake marginal zone samples that contained high levels of nutrients, zoospores were not released immediately. This required approx. 3.5 hours at 5°C, while at 25°C one-half hour was required. These short times, before zoospore release to the water surface occurred, indicated that the zoospores had emerged from mature zoosporangia. If resting spores were involved, several days might be required before zoospore release might occur. This approach also may be useful with sample

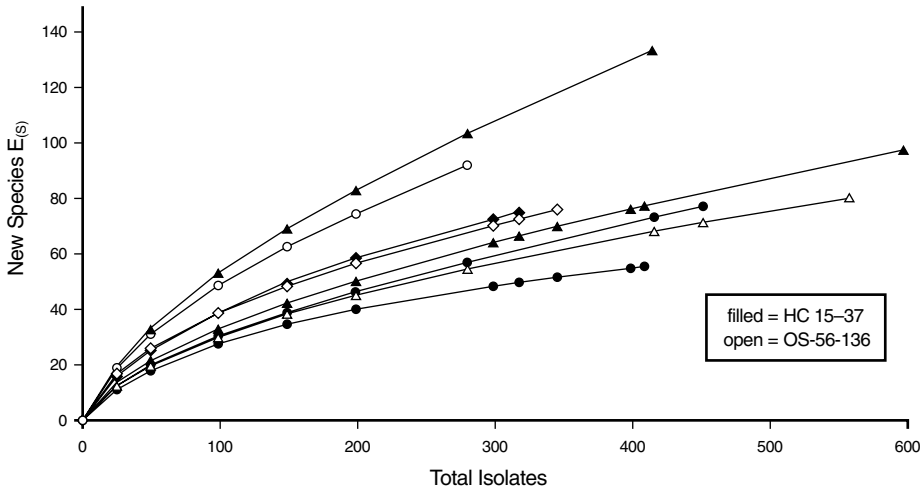


FIGURE 9.4 Use of the particle filtration method for estimating microfungal species richness. Four samples from mixed leaf litter (OS-56-136) and four samples of decayed leaves (HC 15-37), from Costa Rica were analyzed. Rarefaction curves provide information on the number of new species detected per number of fungal isolates examined. $E(s)$ = expected number of species. Reprinted from Bills.⁵¹

dilution, in a MPN format, to develop biodiversity indices based on the zoospores that are recovered, without a major concern for the substrates or hosts being utilized for maintenance of the non-zoosporic stage of the chytrids. Dick⁵³ has made a similar suggestion.

9.2.3.3 Baiting and Microscopic Assessment

Particularly with soils, most studies of chytrids and similar zoosporic organisms have been carried out using baiting, followed by further cultural and microscopic examinations. In these procedures, an environmental sample, if not liquid, is flooded with sterile water and a “bait” substrate is added, such as pollen, fern spores, hemp rope fibers, shrimp exoskeletons, snake skin, or cellophane, all of which, particularly after boiling or autoclaving, are known to attract chytrids.^{38,54,55} Baiting can be carried out using varied observation times. Initial observations are usually made after 3 days, followed by periodic observations over 4–6 weeks. This can be valuable as it allows chytrid succession in the use of particular substrates to be documented. More recent applications of these approaches have been described by Dogma,⁵⁶ Emerson,⁵⁷ Gaertner,³⁷ Letcher and Powell,¹⁸ Perrott,⁵⁸ and Willoughby⁵⁹ as major contributors to work in this area. This approach was used by Willoughby⁶⁰ to quantify the monocentric soil chytrid *Rhizophlyctis rosea* by counting the thalli that grew on cellophane bait in flooded soil samples. This was noted as the first attempt to quantify chytrids in a soil. Chytrids have also been quantified in soil using most-probable-number (MPN) techniques where samples are diluted and an appropriate bait is added to the dilutions. The chytrid population is then estimated based on the highest dilution at which organisms are observed. The MPN technique was applied to the analysis of chytrids by Canter and Lund³⁹ in an investigation of chytrid dynamics in a lake, and in soil by Gaertner.³⁷ Sparrow⁵² has provided an excellent summary of efforts that were given to provide information on the effects of various habitat characteristics on occurrence of chytrids. The work of Dick⁵³ involved the use of dilution/baiting approaches to estimate populations of Saprolegniaceae in samples from the Blelham Tarn area of England. Similar MPN-based approaches have been used in several subsequent studies^{61,40} to provide information on soil management effects on chytrid populations.

Although quantitative procedures based on baiting have provided some knowledge of chytrid presence and abundance in the environment, this approach has distinct limitations. Chytrid zoospores may not be able to colonize the bait due to competition from other microorganisms, as

discussed by Willoughby.⁵⁹ In addition, the actual population of specific chytrids may be underestimated because zoospores will colonize other naturally occurring substrates, under the changed incubation conditions. Barr⁴⁴ has noted that cultural approaches, using baiting, make it difficult to relate the recoveries to the actual substrates being used by the organisms in nature.

9.2.3.4 Serological Methods

Several techniques involving serology and immunology have been developed for detecting chytrids in plants and animals. Delfosse et al.⁶² developed a direct antigen-coating enzyme-linked immunosorbent assay (DAC-ELISA) that was capable of detecting one sporosorus of *Polymyxa graminis* per ELISA well plate. In addition, fluorescein-5-isothiocyanate (FITC)-linked antibodies could be used to detect resting spores in root samples, indicating the potential of this approach for monitoring infection under field conditions. No reaction was observed with common root-associated fungi or *Olpidium*. In further work in this area related to animal systems, Van Ells et al.⁶³ developed an immunohistochemical staining procedure for detecting *Batrachochytrium* in amphibian tissues. In addition, an immunoperoxidase test for *Batrachochytrium* has been developed by Berger et al.⁶⁴ to test for chytridiomycosis in amphibians.

9.2.3.5 Molecular Approaches

Even in molecular analyses of fungi, the chytrids have been considered to be minor parts of microbial communities.²¹ Often, in assessments that are noted to be “fungal,” the zoosporic forms are simply ignored, as discussed by Letcher and Powell.¹⁸ In other cases, the chytrids are used as “outgroups” in studies of fungi in an environment.⁶⁵ Sequence data, involving 18S rRNA analyses, have accumulated rapidly for the filamentous fungi.^{5,66,67} Sequence data also are rapidly becoming available for the chytrids,^{45,25} making it possible to better understand phylogenetic relationships between chytrids recovered from different environments, and to evaluate their relationships with other microbial groups. These sequences also can be used to generate probes for the detection of specific sequences, particularly in the context of MPN dilution to extinction experiments where estimates of populations in particular natural environments can be made.⁴⁰

Molecular techniques have the potential of providing a powerful tool for assessing chytrid (and more general) fungal diversity when nucleic acids from individual active organisms are examined. Unfortunately, in most molecular studies carried out to date where environmental samples (soils, waters) are examined, bulk extraction approaches have been used to recover DNA from these complex microbial communities. The significance of this large and ever-expanding body of information, termed “ecogenomics,”⁶⁸ is compromised by the finding that the method of DNA extraction has the potential of influencing the DNA that is recovered. Steffen et al.⁶⁹ noted that cell extraction versus direct lysis recovered DNA from somewhat different sources, with the cell extraction approach resulting in more bacterial DNA, while the direct lysis approach includes DNA from other sources. In addition, the method of DNA extraction, of itself, can affect the cloned products, based on work with recovery of bacteria.⁷⁰

To the present time, most studies of fungi, where DNA has been recovered from soils and waters, have involved direct lysis approaches.^{65,71,72} As discussed by Bridge and Spooner,⁷³ molecular techniques used in this way do not distinguish between resting and active stages, critical for understanding function, ecology, and biodiversity. Molecular information must be linked with individual active structures that are observed in particular environments to provide molecular information concerning organisms that are active *in situ*.

This level of technology is available; with the use of micromanipulation,⁷⁴ individual active microorganisms can be removed from a particular environment or host and subjected to molecular or physiological analysis, with or without growth. By being able to work with active individual microorganisms, it becomes possible to approach microbial ecology and biodiversity on a unified and consistent basis for both macroorganisms and microorganisms by treating microorganisms as individuals, as is usually done with macroorganisms. To the present time, this has been a major

conceptual and experimental problem that has affected the field of ecology, as discussed by Roberts.²⁷ This linkage, discussed by Klein and Paschke,⁷⁵ makes it possible to circumvent most of these objections and concerns. Work is advancing in this area: Stoeck et al.⁷⁶ combined the use of probes and detailed electron microscopy approaches to evaluate individuals from protistan populations.

This approach also is being used to analyze single AM mycorrhizal spores to assess intrasporal ribosome sequence variability⁷⁷ by analyzing the internal transcribed spacer (ITS) region. The results from these studies suggested that there is substantial intersporal and intrasporal genetic variability. Similar studies of individual fungal conidia have been carried out by Haugland et al.,⁷⁸ while Thomsen and Jensen⁷⁹ used coupled nested PCR to analyze single resting spores from the “*Entomophthora muscae* species complex.” Work at this level of resolution has the potential of enhancing our understanding of chytrid diversity by linking specific active organisms to their molecular information.

Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analyses also may have applications in studies of chytrids and other zoosporic organisms. Using this approach, Nikolcheva et al.⁸⁰ used DNA from known laboratory cultures, including the oomycete *Pythium irregulare*, to obtain information on species richness and community evenness. These parameters decreased with time after initial colonization of leaf disks. These workers also noted that molecular and traditional approaches measured different aspects of the fungi they were studying. Similar approaches might be useful in the analysis of chytrid dynamics in various agriculturally important environments.

The polymerase chain reaction (PCR), when used with appropriate primers in MPN-PCR⁸¹ or reverse transcription PCR (RT-PCR) modes,⁸² should be able to provide valuable information on chytrid diversity. By correcting for gene copy number and detection limits, as discussed by Lozupone and Klein⁴⁰ MPN-PCR results can be standardized to provide estimates of possible organism numbers and responses to agricultural management. As noted by Barr,⁹ “the future of species classification and delineation lies with molecular analysis.” The application of careful molecular analyses, centered on the characteristics of individual chytrids, should make it possible to markedly improve our ability to provide more meaningful assessments of chytrid biodiversity in the future.

9.3 DESCRIPTION OF CHYTRID BIODIVERSITY IN AGRICULTURAL SYSTEMS

9.3.1 CHYTRIDS AS SAPROPHYTES

Chytrids can utilize cellulose, hemicellulose, chitin, and keratin as major polymeric substrates,^{16,52} and these organisms also specialize in the colonization of recalcitrant substances such as pine pollen. Despite the varied and important roles that these organisms may play in the soil environment, little is known about their ecology or abundance in nature, critical aspects of diversity.⁸³

Chytrids are versatile processors of organic matter, particularly of complex polymeric substrates, that can have both positive and negative effects on agricultural production systems.

Much of the early work in this area has been summarized by Sparrow.¹² From a beneficial standpoint, chytrids contribute to the degradation of organic materials, important in biogeochemical cycling. From a negative standpoint, if environmental conditions are appropriate, chytrids can affect the stability and quality of many organic products. In this context, they often serve as primary colonizers, and following their activity, other decomposers may be able to more rapidly process complex substrates. Harder⁵⁵ documented chytrid occurrence in a wide variety of terrestrial environments; soil disturbance led to increased chytrid occurrence, particularly of *Nowakowskiella* and *Rhizophlyctis*. Remy⁸⁴ also observed increased occurrence of these genera, as well as of *Rhizophydium*, in disturbed soils. Most of these earlier studies were carried out with “baiting,”

using insect wings, seeds, plant parts, and ant larvae, usually autoclaved or boiled before addition to the desired environment.

There are only a limited number of studies where direct observation without baiting has been used, and these have been carried out primarily in aquatic environments, where particulate matrices do not unduly interfere with microscopy-based assessments. Some of the more interesting studies are those of Sherffel,¹⁴ in which zoospores were characterized, while Sparrow⁸⁵ studied the associations of Monoblepharidales with plant materials. In this latter study a temperature effect also was noted; with incubation of materials at 8–11°C, sporangia were formed, while at 21°C sexual reproduction occurred.

Chytrids also have direct impacts on agricultural production systems via their participation in anaerobic degradation processes in a wide range of ruminants where they function as saprophytes. These anaerobic fungi, first described in sheep by Orpin,^{86,87} have been found to occur widely in fore- and hindgut fermenting animals.⁸⁸ *Neocallimastix*, one of the most intensively studied of these anaerobic chytrids, has a unique life cycle, involving attachment of the polyflagellated zoospore, followed by germination, formation of the rhizomycelium, zoosporangium maturation, and eventually, by zoospore release (fig. 9.5). These unique zoosporic fungi depend on hydrogenosomes for processing their nutrients and creating chemiosmotic gradients. Their metabolism involves a pyruvate:ferridoxin oxidoreductase and linked hydrogenase.⁸⁹ Their main function appears to be the degradation of plant cell wall materials by elaboration of a wide range of hydrolytic enzymes. Other important genera contributing to these plant material degradation processes include *Caecomycetes* and *Piromyces*⁹⁰; polycentric species have been observed to be more effective than monocentric species.

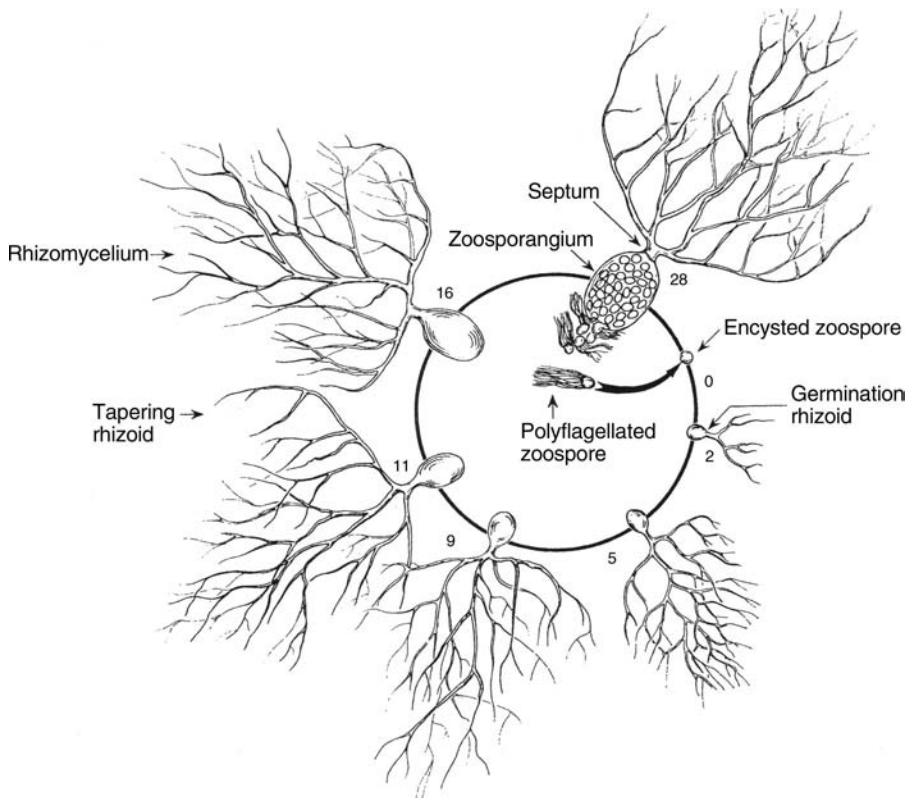


FIGURE 9.5 A life cycle of the anaerobic monocentric fungus *Neocallimastix harleyensis*, depicting the initial attachment of the polyflagellated zoospore, and the successive development, resulting from the formation of the zoosporangium and the release of zoospores. Numbers note hours after zoospore encystment. Reprinted from Trinci et al.⁸⁸

Lee et al.⁹¹ found that the anaerobic fungi were responsible for most of the plant cell wall degradation that occurs in ruminant digestive systems, based on microcosm studies where the functioning of microbial communities with varied bacterial, protozoan and fungal activities were evaluated.

9.3.2 CHYTRIDS AS BIOTROPHS

Many chytrids are active parasites infecting fungi, algae, higher plants, and animals.^{44,92,16,93} Of special interest is the genus *Spizellomyces*. *Spizellomyces* species are widespread in soils⁹⁴ and have been reported to parasitize the oospores of *Sclerospora sorghi*,⁹⁵ *Peronospora tabacina*,⁹⁶ the chytrid *Synchytrium endobioticum*,⁹⁷ and soil nematodes.⁹⁵ In addition, *Spizellomyces* infects the spores of arbuscular mycorrhizal (AM) fungi^{93,98} and has been hypothesized to reduce AMF populations in the field.⁹⁸ *Spizellomyces kniepii* also has been found to transmit spiroplasma-like organisms between AMF spores.⁹⁹

9.3.2.1 Plant Interactions

Chytrids play important but often less appreciated roles in the functioning of plants.^{94,100} Potato wart disease (also called black wart), caused by *Synchytrium endobioticum*, can have major effects on crop yields and tuber quality. In 2000, an occurrence of potato wart disease led to a quarantining of potato shipments from Prince Edward Island, Canada.¹⁶ It was a small outbreak that had important consequences, because *Synchytrium* spores can stay viable in the soil for 30 years and the disease makes potatoes unmarketable. In North America it is usually present only in Newfoundland, but it is also present in Europe. Molecular tests to detect *S. endobioticum* were rapidly developed. Levesque et al.¹⁰¹ used sequences provided in James et al.⁴⁵ to develop chytrid-specific primers for PCR-based detection of *Synchytrium* in infected potatoes.

In addition, chytrids such as *Olpidium* are found in the roots of a wide variety of grasses. As summarized by Campbell,²³ zoospores of *Olpidium*, and also of the Plasmodiophorales *Polymyxa* and *Spongospora*, can serve as vectors for plant viruses. As discussed by Powell,¹⁶ a major infection mechanism involves virus release to the soil, following decomposition of the diseased root. The virus then becomes associated with the zoospore surface and is transmitted to the plant cytoplasm when the zoospore penetrates the plant. Recent studies¹⁰² indicate that this process of virus sorption to the zoospore surface is mediated by oligosaccharides that appear to contain mannose and/or fucose, based on studies of the cucumber necrosis virus.

The major approaches that have been used to control chytrids include chemical disinfection of soils, quarantining of products from infected areas, and the application of chitin to soils.¹⁶ In this latter approach, infected soils are amended with chitin, derived from crabshells, leading to the development of increased populations of chitinolytic microorganisms. This approach has been used successfully to decrease potato wart occurrence¹⁰³ due to the susceptibility of chitin-containing resting chytrid spores to chitinases released by streptomycetes.

Work is in progress to attempt to develop control agents for other zoosporic pathogens. Antibiotics from a *Streptomyces* are being evaluated to attempt to control the oomycete *Pythium*, the causative agent of red rot disease in *Porphyra* spp. Woo et al.¹⁰⁴ and Jensen et al.¹⁰⁵ have described a new antibiotic that protects sea grasses against zoosporic fungi. Such chemicals may prove useful as the basis for development of specific plant protection approaches to safeguard against chytrid predation. Quarantining of plant materials infected with zoosporic organisms can play an important role. As discussed by Kanyuka et al.,¹⁰⁶ the resting spores/cysts of *Polymyxa*, particularly, can remain viable in the soil for decades, once infected plant materials might degrade in the soil.

9.3.2.2 Animal Interactions

The range of animals that can be parasitized by chytrids has been discussed by Powell.¹⁶ *Catenaria*, as an important example, attacks rotifers, tardigrades, midge eggs, and bark beetles, as well as

nematodes. This organism encysts on the nematode cuticle before actually penetrating the susceptible organism.¹⁰⁷ Parasitic chytrids may be able to serve as control agents against these plant pathogenic nematodes. Other chytrids of possible interest in terms of biocontrol include the genus *Coelomyces*, parasitic on the mosquito vector of malaria, where a crustacean is involved as a secondary host in the chytrid's life cycle.¹⁰⁸

Although only of indirect interest in terms of the major agricultural production systems, the chytrid *Batrachochytrium dendrobatidis* is of increasing concern.^{92,109,110} Isolates of the organism appear to belong to a recently spread asexually reproducing clone.^{25,92,111} A strong link was detected between chytrid presence and mortality of a wide range of amphibians. However, the total range of factors that have led up to the increased occurrence or at least participation of chytrids in these amphibian mortalities remains to be established. Population declines of amphibians have occurred in what have been considered to be "pristine areas," although weather variations were considered to possibly play a role in these amphibian losses.¹¹² An additional factor that might lead to the increased dispersal of this disease is the possible importation and exportation of infected amphibians from continent to continent, and the differential susceptibility of different amphibian species to these chytrids.

The possible changes that might be occurring in specific habitats, leading up to such declines, however, may be more subtle. There are a wide range of secondary factors, such as UV and pesticides,¹¹³ temperature,¹¹⁴ and other anthropogenic factors¹¹⁵ that may be mediating or facilitating the increased impacts of chytrids on amphibians that are occurring in various parts of the world. In addition, such "stressors" may have different effects at lower versus higher concentrations, and also differential effects on the biotic components (chytrids, amphibians, other organisms) of the particular ecosystem, important aspects of hormesis.¹¹⁶ Careful experimentation will be required to establish the nature of these interactions, and to better understand the role of specific management alternatives on disease occurrence.

In addition to these concerns for chytrids as pathogens, an important positive application of a zoosporic organism in agriculture is the addition of *Schizochytrium* spp., a thraustochytrid with a motile heterokont zoospore, to dairy cow feeds to increase concentrations of conjugated linoleic, ducosohexaenoic, and transvaccenic acids in milk.¹¹⁷ This organism also is being added to a wide range of feeds, including those used for fish.^{118,119}

9.3.3 MICROBIAL INTERACTIONS

Other chytrids actively parasitize a wide range of microorganisms, interactions that primarily may have indirect subtle effects on agricultural production systems. Many are parasitic on algae. These include the carefully described attack of *Asterionella* by *Rhizophyidium*¹⁰⁷ that can result in massive lysis of algal blooms¹⁶ with subsequent release of nutrients to water.

Other chytrids have been implicated in the parasitism of filamentous fungi in soils. Chytrids can specifically attack chlamydospores, resulting in the loss of these fungal structures.¹²⁰ Chytrids also can attack the spores of AM fungi,⁹⁸ although the susceptible AM spores appear to be those that are damaged or stressed, and thus this represents more of a saprophytic than a biotrophic relationship. In addition, *Rozella allomyces*, which parasitizes *Allomyces* sp., and *Caulochytrium protosteloides*, which parasitizes hyphomycetes, are important in agricultural soils, as summarized by Barr.¹⁰ *Caulochytrium* is considered as a biotrophic haustorial mycoparasite. It is a unique chytrid genus in that it produces both sessile zoosporangia and aerial sporangocarps.¹²¹ As with the rhizoids of other saprophytic Chytridiomycetales, nuclei are absent from these haustoria involved in parasitic metabolic exchanges.

9.3.4 SUMMARY: BIODIVERSITY IN AGRICULTURAL SYSTEMS

In terms of all-over parasitic impacts, the low levels of chytrids observed in most agricultural systems do not appear to lead to the death of most hosts. They may, however, lessen efficiency and possibly

diminished the ability of host organisms to maintain themselves in particular environments, particularly when they might be subjected to additional physiological or environmental stresses. The possible impacts on nematodes are more difficult to assess; the chytrids may deserve more credit in controlling nematodes—we are limited by our ability to determine microscopically that these interactions are occurring. In addition, by utilizing nonliving or living hosts as sources of organic nutrients, the chytrids contribute to the microbial loop, in which organic matter is mineralized and nutrients are made available again for use by primary producers, instead of being transferred to higher trophic levels, a major potential impact in terms of agricultural productivity. The impacts of chytrids on virus transmission are well documented. The major challenge is developing control strategies to be able to limit the effects of chytrid-mediated virus transmission to susceptible crops.

9.4 STRUCTURAL AND FUNCTIONAL RELATIONSHIPS OF CHYTRIDS

The chytrids represent an example, *par excellence*, of a microbial group where structure and function are inextricably linked. The major advantage that the chytrids have, based on their possession of a motile state, in comparison with many of the filamentous fungi, is the ability to find, attack, and exploit hosts or substrates over relatively large distances. These substrate-seeking processes are refined to the point where even differences between edges and surfaces of a substrate, such as cellulose, can result in major differences in the density of colonization by chytrids.¹²⁰

The broader aspects of structure–function relationships have been emphasized by Emerson.¹²² He noted that the chytrids, being especially sensitive to differences in their environment, respond with refined morphological variations that can be of major importance to understanding the strategies used by these unique organisms.

Major structural and functional strategies include the following:

1. By having a zoosporic stage, genetic information can be moved according to sensing and directional processes. Most zoospores will develop near the parent thallus, perhaps over a distance of 2 cm. In addition, zoospores can be moved passively by water currents, etc. They can develop on less than optimum substrates but they have the morphological plasticity to develop small sporangia in such less favorable conditions.
2. The zoospore, after attachment to the host or substrate and development of its thallus, particularly within a host, can be protected from predation and environmental stress.
3. Reproduction can occur involving either asexual or sexual cycles. For many chytrids, only asexual processes have been observed to date. The ability to carry out life cycles without observable sexual processes, as occurs with *Neocallimastix* and *Physoderma*, can lead to accumulation of mutations, and the appearance of what can be considered to be “new species.” This asexual replication does, however, result in a maximized allocation of resources to new vegetative growth and may allow more effective maintenance in a dynamic, high turnover environment such as the rumen.
4. The ability to generate motile zoospores in a protected structure (the sporangium) allows their release to the environment only when the zoospores are mature and prepared to move quickly to new areas where resources may be available for exploitation.
5. Spores, sporangia, and zoospores also can be moved over great distances by passive movement of soils and plant materials. In addition, the ability of walled resting spores, such as those formed by *Synchytrium*, to survive in soils, literally for decades, increases the opportunities for dissemination to new habitats through erosion and soil disturbance.

These structural and functional attributes of chytrids present challenges when attempting to document chytrid biodiversity. Having structures that are often hidden in other organisms, and to

some extent unobservable, makes it more difficult to document with confidence the biodiversity of these organisms. Long-lived structures, such as resting spores, may simply be present in an environment but not contribute to functional biodiversity under a specific set of conditions, due to intrinsic and/or extrinsic limiting factors. As noted earlier, when environmental conditions might change, organisms present as resting structures may be transformed to active forms, resulting in their being able to contribute to functional diversity.

9.5 FACTORS CONTROLLING CHYTRID BIODIVERSITY

Most of the literature that is available does not directly address this concern. As noted earlier, most literature is directed toward surveys using indirect baiting approaches that do not allow the specific assessment of *in situ* functional diversity. These studies do, however, suggest that the chytrids respond to variations in water, nutrient availability, and physical disturbance. Remy⁸⁴ and Harder⁵⁵ noted that *Rhizophlyctis* populations increased in disturbed soils. Additions of organic matter (insect and animal remains, etc.) to soils under moist, aerobic conditions can lead to a greater variety of chytrids.⁵² Habitat factors that influenced chytrid responses included light, temperature, oxygen, pH, altitude of habitats, and substrate characteristics.

More recently, Lozupone and Klein⁴⁰ studied the effects of soil disturbance on general chytrid populations and *Spizellomyces* in shortgrass prairie soils, where cultural and molecular techniques were used. The MPN-based estimate of chytrid propagule numbers in the various environmental samples are summarized in table 9.1. A pairwise comparison with the students' t-test indicated that significantly lower populations of pollen-metabolizing chytrids were present in the native, nitrogen-amended plot than in the disturbed control plot ($p < 0.05$). Mineral nitrogen also has been shown to cause decreases in filamentous fungal hyphal lengths in soils.^{123,124} Higher populations of pollen-metabolizing chytrids were suggested to present in the disturbed-control plot, in comparison with the native-control plot estimates. *Spizellomyces* population estimates in the various environmental samples (table 9.1) suggest that these were only a minor part of the entire pollen-metabolizing chytrid community, but the *Spizellomyces* populations appeared to increase in response to disturbance in the control plots ($p < 0.1$).

TABLE 9.1
Estimated Chytrid and *Spizellomyces* Populations in Uncultivated and Cultivated Shortgrass Steppe Soils, With and Without Mineral Nitrogen Amendments. Cultural MPN Results and 95% Confidence Intervals Using a Pollen Bait are Noted

Organism group	Soil type used	MPN estimated mean per gram soil	95% confidence interval
All chytrids	Native control	1315	263–5934
	Native nitrogen ^a	877	198–3675
	Disturbed control ^a	4605	1123–11520
	Disturbed nitrogen	3165	719–11320
<i>Spizellomyces</i>	Native control	135	17–521
	Native nitrogen	469	16–1881
	Disturbed control	673	83–2606
	Disturbed nitrogen	469	16–1881

^a Mean population estimated per gram of soil is significantly different for these two treatments at $\alpha = 0.05$.

Reprinted from Lozupone and Klein.⁴⁰

MPN-PCR also was used to estimate the number of *S. acuminatus* and *S. kniepii* genetic units in these soils using primer pairs developed specifically for detection of these closely related organisms.⁴⁰ Based on use of this approach, a longer-term effect of disturbance on chytrid populations was suggested, while no N-amendment effect could be observed.

Chytrids appear to respond in a different way to disturbance than the filamentous fungi. In related studies carried out at the same sites,^{123,124} soil disturbance resulted in decreased levels of total and active filamentous fungi. The possible increase in chytrid populations that appear to occur at these same sites, however, is not surprising, given the functional strategies used by this group. Disturbance, especially by cultivation, often increases the concentration of nutrients available in soil and decreases the physical heterogeneity of nutrient resources. Chytrids can rapidly reproduce, releasing highly chemotactic zoospores when conditions are favorable. These characteristics could allow them to quickly exploit the nutrients released in response to disturbance, including hyphae released from disrupted filamentous fungal networks, increasing the overall chytrid populations, as observed by Lozupone and Klein.⁴⁰

9.6 FUTURE RESEARCH NEEDS: BIODIVERSITY AND THE CHYTRIDS

What factors need to be considered in further work on the biodiversity of these organisms?

First, there is a continuing need to work with direct observation and linking of molecular information with specific functionally active structures that can be observed *in situ* and then recovered for characterization by molecular techniques. Baiting and MPN-type approaches, while providing some information, are only the first steps in developing a more substantial grasp of the biodiversity of these organisms. To meet this need, direct recovery of individual active organisms from the particular environment being studied, followed by molecular analyses, should make it possible to link molecular technique-derived information to fungal structures, particularly chytrids that are observed to be active in soils or other environments.

Second, there is a need to assess the direct and indirect effects of various stressors and trace environmental components, such as occult nitrogen and acid rain components, on the functioning of chytrids as saprophytes, and particularly as biotrophs. The same stressing agent may have different effects at low and high concentrations. This phenomenon of hormesis¹¹⁶ may be having subtle effects, especially on amphibian systems. The observed effects of chytrids may only be secondary responses to other factors that have changed in these systems.

Third, there is a need to document and quantitate the specific contributions of chytrids to the functioning of ecosystems. This will require, at a minimum in a first level of experimentation, the use of constructed microcosms, with and without various biotic components present, as carried out by Lee et al.,⁹¹ or the application of specific chytrid inhibitors (if they can be found) to be able to begin to assess specific chytrid contributions to the structure and function of ecosystems.

9.7 SUMMARY

Chytrids, and the various nonfungal zoosporic organisms with similar ecological strategies, present unlimited opportunities for the curious observationist and particularly for the student of managed agricultural systems. These organisms, with their heterotrophic nutrition, impose small but not insignificant energetic and nutrient cycling-related costs on ecosystems. However, in most studies of fungi in soils, as an environment of major agricultural concern, too often chytrids simply are not looked for, either directly or in terms of molecular analyses. These organisms, considered to be difficult to study in terms of direct observation of active forms, have not been subjected to sufficient in-depth investigations to allow a realistic assessment of their *in situ* active biodiversity.

Their potential impacts, particularly in terms of biodiversity, warrant greater appreciation and more detailed examination.

Other than in striking cases where chytrids have significant economic impacts, such as of potato black wart disease or virus transmission to susceptible plants, which often occur in monoculture environments created as a part of intensive agricultural production systems, the chytrids and other zoosporic protists appear to have only subtle effects on the structure and function of most plant communities. In addition, the ability to control or manage chytrid-mediated diseases is limited, other than by the use of chemical fumigants, quarantining, or possibly chitin additions to soils.

In terms of biodiversity assessment, it must be recognized that chytrids are more than merely “simple microscopic, primarily aquatic phycomycetes,” as noted recently,¹²⁵ too often simply omitted from investigations of fungi in complex systems. Their occurrence, distribution, and function in soils, as an environment of major concern, are poorly understood, as discussed by Letcher and Powell.¹⁸ Chytrids show a complexity of structural development and refined ecological strategies that affect all areas of ecological science. Their biodiversity, and its control by various environmental factors, is a critical aspect of better understanding their all-over effects on agricultural production systems.

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10 Diversity of Arbuscular Mycorrhizal Fungi

Philipp Franken and Eckhard George

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10.1 INTRODUCTION

The phenomenon “Mycorrhiza” comprises all symbiotic associations of soil-borne fungi with roots or rhizoids of higher plants and was first introduced into the literature by Frank (1). The group of fungi and plants, which are involved in the interaction, determines the type of mycorrhiza they form (2). Arbutoid and monotropoid ectendomycorrhizas, as well as the ericoid and orchidoid endomycorrhizas, can only be found in a few plant species and are, therefore, restricted to certain ecosystems. In contrast, ectomycorrhizas are established between a great variety of mostly Basidiomycota and the roots of many woody plants. This symbiosis is very common in forest ecosystems in the temperate zone and can be applied to plant production systems in tree nurseries (3). The most widespread type, however, is represented by the arbuscular endomycorrhiza, and this chapter concentrates on this type of symbioses, because the plant hosts include the most important crops used in agri- and horticulture.

Arbuscular mycorrhizal (AM) fungi are ancient microorganisms. Fossil data and molecular phylogenetic analyses indicate that they appeared between 460 and 400 Myr ago during the Ordovician-Devonian (4–6). During this period the plants started to colonize the land and it is tempting to speculate that the AM fungi might have been essential for this process (7,8). Nowadays they are integral components of most terrestrial ecosystems (9), and some 80% of terrestrial, vascular plant families act as hosts for the fungal endosymbionts (10). Despite the considerable radiation of the plant hosts (approx. 225,000 species), only about 130 species of AM fungi are described. It was recently suggested that these symbiotic microorganisms should be removed from the Zygomycota as they represent their own new phylum, the Glomeromycota with four different orders (11). Two of these orders, the Glomerales and Diversisporales, contain the genera *Glomus*, *Gigaspora*, *Scutellospora*, and *Acaulospora*, to which most isolates have been assigned.

The small number of different fungal species might suggest that their biodiversity is limited. However, we would like to show in the following that this is not the case. The variation at the morphological, physiological, and genetic levels is rather high and this results in a functional diversity that has an important impact for ecology and applications in plant production systems.

10.2 GENOTYPIC VARIATION

Genotype analysis of the AM fungi has been started by estimating the size of their genomes. Estimates vary between different AM fungal species ranging from 250 Mb to 740 Mb (12) or from 120 Mb to 1060 Mb (13), which is rather high for fungal organisms. However, in the fungus *Glomus intraradices*, Hijri and Sanders (14) showed that the haploid genome size was only around 15 Mb, which is on the lower limit for eukaryotes. The difference in these genome size estimates shows that the variation of this feature among AM fungi is immense. Interestingly, these differences are unlikely to be due to different levels of ploidy because in both studies the genome size did not differ significantly from the nuclear DNA content, indicating that the nuclei are always haploid.

The first AM fungal genes that were analyzed in more detail encoded the 18S ribosomal RNA (15). This opened up the possibility of using molecular PCR-based methods for phylogenetic analyses and for identification of AM fungi (15,16). Nowadays, these techniques are applied to the whole rRNA gene cluster and are also used for estimating the genetic variation of AM fungi in natural and agricultural soils (17–21). One interesting result of these analyses was that sequences of rRNA genes did not only differ between species and isolates of AM fungi. In addition, applying these methods to single spores of AM fungi showed that intra-isolate variation of rRNA genes also exists (22–28). The reason for this can be explained in two different ways. On the one hand, the sequence variation might be due to differences between copies of the rRNA gene cluster. It has been shown at least for *S. castanea* that the genome contains approximately 70 copies of this locus (29). On the other hand, as coenocytic organisms, AM fungi contain huge numbers of nuclei in one spore (30) and the different sequences could represent alleles of one locus among the genomes inside of these spores. That this is the case could be shown by using specific probes that recognized two different ITS2 sequences among nuclei of one spore (31).

Sequence information about rRNA encoding gene clusters is a suitable tool for research about the phylogeny, the taxonomy, and the biodiversity of organisms. However, increasing our knowledge about the function of AM fungi needs the isolation and analysis of protein-encoding genes. Such genes were first identified by PCR using genomic DNA or RNA from spores as template (32,33) or in a cDNA library obtained from the symbiotic stage (34). The variation of such sequences among different AM fungi has been investigated for genes encoding chitin synthases, beta-tubulin, actin, the translation elongation factor EF1alpha, or H⁺-ATPases (35–42). In some cases, the variation within one isolate was also investigated for such protein-encoding genes. An analysis targeted to this specific question was carried out by Kuhn et al. (31). Among 15 sequences of the BiP gene in *G. intraradices*, they detected a similarity of between 92 and 99%. Because the genome is haploid and rather small (14), these sequences very probably represent alleles of one locus or a few loci distributed among the different nuclei of this isolate. This is also the case for the actin and the EF-1alpha gene, because the sequence differences were also rather small (39). Moreover, Southern blot analysis of the EF-1alpha gene showed that at least in *Gigaspora rosea* it is a single copy gene (Sieh and Franken, unpublished). The basis for the sequence variation seems to be different for P-type H⁺-ATPase genes. They probably belong to members of a gene family, because they have different evolutionary origin (41) or show differential gene expression (42). In the case of the beta-tubulin genes, probably both are true. One isolate harbored very closely related sequences and sequences with higher variations (38). Sequences of different evolutionary origin have to be handled with care, because AM fungal spores are often the host for contaminants, as has been discussed (40,43). In case of the beta-tubulin genes, one of the copies appears to be from a plant or an oomycete (38), but in this the sequence is very probably derived from the AM fungal genome, because the expression of this copy was detected in hyphae from root organ cultures, which are free of contaminants. Moreover, among cDNA fragments from *G. intraradices* extraradical hyphae harvested from *in vitro* cultures, a family of glutathionein genes was detected, where also one member showed homology to plant and not to fungal genes (Sieh and Franken, unpublished). A

final answer to this problem will be given in the close future, when the total genome sequence of *G. intraradices* is available.

So far we have mostly considered the genetic differences among isolates of different genera or species. However, a recent study also highlights the huge molecular variation among isolates of one AM fungal species, *G. intraradices*, originating from a single field (44). The study shows very large genetic differences among isolates, a level of genetic variation that might typically be seen among plant populations on a continental scale. Furthermore, the isolates differed greatly at the phenotypic level as well, and due to the design of the experiment, those phenotypic differences are likely to also have a genetic basis. This study outlines the need to consider diversity in AM fungi at genus, species, and within species levels.

In summary, the genotypic diversity of AM fungi is rather high between species and can also be detected among the genomes of one isolate. Variations among isolates are mirrored in morphological, physiological, and functional differences, as described below. Whether the sequence variation between the nuclei of one isolate has consequences for the interpretation of gene expression analyses is questionable. Using cDNA array technologies, the different transcripts present as corresponding cDNAs in the probe will probably hybridize to one target on a membrane and variations in gene expression will be blurred. Even the published RT-PCR experiments have very probably detected the majority of copies in one experiment, because sequence variations are low between different alleles. If one would want to take the different alleles into account, then very specific primers would have to be designed that are able to distinguish subtle sequence differences. However, differences in the expression between two alleles will be very rare, because it needs a nucleotide exchange precisely at a very sensitive site of the promoter region and the statistical probability for such an event is rather low (unless paralogs of a gene have diverged under strong positive selection after a duplication event). More important than for differences in the expression are probably the consequences for the functional analyses of genes. Differences that lead to changes in the amino acid sequence might lead to series of corresponding proteins. Such protein varieties will be present in parallel in one huge compartment reaching from the spore to the arbuscule, harboring slightly different characteristics like pH or temperature optimum. If this is the case, it could explain the flexibility of a single AM fungal isolate to changing environmental conditions.

10.3 PHENOTYPIC VARIATION

The most obvious difference between AM fungal species is the morphology of their asexually formed chlamydospores. They can vary in size (50–600 μm diameter), in color and in cell wall structure, as well as in the number of nuclei they contain. These morphological features are the basis for their taxonomy (45,46). In addition, spores of some species contain so-called bacteria-like organisms (BLOs) as has been intensively investigated in the species *Gigaspora margarita* (47). However, BLOs appear to be totally absent in the closely related *G. rosea* (48,49).

All AM fungi are obligate biotrophs. This has been always ascribed to their inability to take up carbohydrates as saprophytic fungi do. However, it has turned out that this is not the case (50). The lack of lipid biosynthesis when living in the nonsymbiotic state has been suggested to more likely account for their biotrophy (51). Following germination, AMF show only a very limited hyphal growth without a plant (52,53). Interestingly, in the case of the genera *Glomus* and *Acaulospora*, germination occurs also from old hyphae, while in the case of *Gigaspora* and *Scutellospora*, only the chlamydospores function as propagules. This has clearly consequences for the formulation of inocula (54).

Spore germination of certain AM fungal species can be induced by abiotic factors like temperature and humidity (55), while the spores of others are not able to germinate for a certain period (e.g., 56). A particular feature of the two genera *Gigaspora* and *Scutellospora* is the development of auxiliary cells, which form on the asymbiotic hyphae independently of host roots. These structures

have been suggested as possible infective propagules (57) or reminiscent of spores (45), but their true role in the life cycle of AM fungi is still not clear.

A clear difference between AM fungal species has also been detected on the molecular level. Investigating spore content showed that *G. rosea* does not harbor detectable amounts of RNA immediately after isolation from soil. Storage for about one week in water at 4°C, however, resulted in RNA accumulation to rather large amounts (33). In contrast, spores or sporocarps of *Glomus mosseae* contain low amounts of RNA immediately after isolation from soil and keep this amount constant for at least 3 months (Bütehörn and Franken, unpublished). During asymbiotic germination, no significant changes in RNA accumulation pattern could be observed in *G. rosea* and *S. castanea*, while differential occurrence of cDNA fragments have been observed in *G. mosseae*, indicating regulation on the transcriptional level for this fungus from the Glomales (58,59). The asymbiotic hyphae branch in the vicinity of a host entering the so-called presymbiotic stage (60). This branching can be induced not only by root exudates (61), but also by numerous soil bacteria and fungi (62). Some of these experiments, where AM fungal species were compared, detected contrasting responses. For example, cell wall-associated compounds stimulated the growth of *G. margarita* hyphae, but had no effect on *Gigaspora gigantea* (63) and a *Bacillus subtilis* strain induced branching of *G. mosseae*, but totally inhibited the hyphal development of *G. rosea* (64).

The symbiotic stage of the interaction starts with the development of an appressorium upon physical contact of the fungus and the root. After entering the root from this appressorium, hyphae colonize the cortex, but never enter the central cylinder. Although the specificity of the AM symbiosis is rather low, the intensity of colonization can be very different depending on the type of plant and fungus, which are interacting. Two general modes of colonization can be observed. In the beginning, hyphae first grow intercellularly or cross outer cells with linear or simple-coiled hyphae (65). Both possibilities have been observed in different plant–fungal combinations, but it is not clear, if it only depends on the plant genotype or if it is also a result of fungal diversity, since comparing analyses has never been carried out. In a second step, when the hyphae reach the inner cortex, they develop arbuscules. In the Arum type, the hyphae are starting from intercellular hyphae to penetrate the cell wall and to build up one highly branched arbuscule per plant cell. In the Paris type, the intercellular phase is absent and hyphae grow from cell to cell where they develop large coils with small intercalated arbuscules. The type of mycorrhiza that is formed seems to be determined by the genomes of both partners, and is not under environmental control (66). This phenomenon has been analyzed in more detail in the tomato: Using different inocula, *G. intraradices*, *G. mosseae*, and *Glomus versiforme* formed the Arum-type, while *G. margarita*, *Glomus coronatum*, and *Scutellospora calospora* formed the Paris-type (67). The same authors included in a similar experiment the tomato mutant *rmc* and again they observed great differences: Some AM fungi were arrested at the appressorium stage, a second group could penetrate the root surface, but are stopped in the outer cortex cells. Only one fungus, *G. versiforme*, developed a relatively normal mycorrhizal symbiosis (68). Other typical structures of AM fungi are vesicles, which are only formed by members of the Glomerales and not by the Diversisporales, and are considered to be storage organs (69).

In parallel to the colonization of the root cortex, AM fungi also spread into the surrounding soil. One important function for the fungus of these extraradical hyphae is the development of new spores, and the pattern of this is also different between AM fungal species and can be used for taxonomy (45). Also, the mycelium explores the environment for nutrients and the transport of phosphate, nitrate, and trace elements from the fungus to the plant (70) is the most obvious function of the AM symbiosis. Numerous investigations have shown diversity in uptake of phosphate and zinc (71), nitrogen (72,73), or iron (74). Variation in P nutrition was, for example, explained by the metabolism of the fungal mycelium. *G. rosea* and *Glomus manihotis* showed significant differences in the accumulation of polyphosphates and in the activity of the alkaline phosphatase (75,76). Interestingly, in this respect AM fungi are able to display a functional complementation: *Glomus caledonium* is very efficient in the transport of phosphate from sites more far from the plant, while *S. calospora* preferentially obtained the nutrient in close vicinity to the root (77). Another experiment

tried to correlate P transfer efficiency and plant gene expression. Comparing seven different fungal species, *G. mosseae* showed the highest rate of P uptake and strongest inhibition of a phosphate transporter gene of *Medicago truncatula*, which is probably responsible for the P uptake under nonmycorrhizal conditions (78). Doing the same experiment with the tomato, the expression of the corresponding gene was constantly low, independent of the fungal species. Further experiments indicated a large diversity of mechanisms of P-uptake pathways in different fungal–plant combinations (79). In the combination flax–*G. rosea*, for example, there was a 60% increase in P uptake in mycorrhizal compared to nonmycorrhizal plants, but only 9% of this increase was due to the transport via the fungal mycelium. It seems to be that in this combination the normal plant P-uptake via epidermis and root hairs was induced by the symbiont. Another result of this study was that the growth-promoting effect by AM fungi does not necessarily correlate with the P-uptake induction, which might at least partially be due to different demands of the fungus for carbohydrates. That this can be the case has been shown among three isolates of *G. mosseae* (80). While BEG 54 was a strong and BEG 55 a moderately strong C sink, BEG 12 gave similar results concerning C partitioning as the noninoculated control plants, showing that the diversity of AM fungi also differentially affects the carbon budget of the plant.

Another important function of the AM symbiosis for the plant is the improved tolerance to abiotic stress situations (81). The influence of arbuscular mycorrhizal fungi on the performance of plants on heavy metal contaminated soils has been the topic of many studies and variation in plant performance with different AM fungi was clearly shown. In this respect, the most important factor seemed to be the origin and source of the inoculum. Those isolates that were obtained from heavy metal contaminated soils were the most effective (82–86), indicating the ability of AM fungi to become adapted to high stress environments. However, if such isolates are cultivated on heavy metal-free soils for inoculum production, they lose their capacity to transfer tolerance to plants (87). Concerning the mechanism, AM fungi display, on the one hand, a certain level of heavy metal tolerance, which is mirrored by their germination frequency on water agar containing different concentrations of heavy metals (88). On the other hand, the mycelium is able to bind heavy metals (89) and the specific site of this binding can differ between isolates of AM fungi (90). This binding is probably the reason that heavy metals accumulate in roots, but are not transported to the shoots (91). The binding capacity of the mycelium, however, may not be sufficiently large to contribute significantly to effective protection of plants on heavily polluted soils.

The availability of water is an important factor for plant growth and drought is limiting plant production in many regions of the world. AM fungi are able to improve plant performance under such adverse conditions (92,93). Certain isolates show differences in this respect and this also seems to depend on the site of isolation (94,95). A comparison of seven *Glomus* isolates in symbiosis with lettuce showed that these differences are not related to decreases in photosynthesis or water use efficiency, but to higher transpiration rates, levels of leaf conductance, and proline content (96), and perhaps at least partially to an improved uptake of water (97).

In contrast to the investigations of the influence of AM on abiotic stress, the effects of the interaction between roots and different AM fungal isolates on pathogens has only been rarely compared. One study showed that *G. mosseae* reduced the disease index of tomato roots infected with *Phytophthora parasitica*, while *G. intraradices* did not (98). Interestingly, the induced resistance was correlated with the expression of a specific beta-1,3-glucanase (98). However, this enzyme was only locally but not systemically activated, although bioprotection was observed in the whole root (99).

Differences in the phenotype and the function of mycorrhiza must be mirrored at the molecular level. Two studies on phenotypic variation among isolates of a *Scutellospora pellucida* and among *G. intraradices* show clearly that within species variation in spore color, shape, extraradical hyphal growth rates, and spore production has a genetic basis (100,44). Despite the fact that neither of those studies investigated the genes involved in that variation, they clearly show that the pursuit of the molecular basis of variation in these traits is warranted. Gene expression analyses have been

carried out in various plant–fungal combinations (101,102,59), but direct comparisons can only rarely be found in the literature. Enzyme activities have been measured in the interaction of *Ziziphus xylopyrus* with six different AM fungi and a correlation was detected between beneficial effects on plant growth and level of the biochemical parameters (103). Superoxide dismutase activity could be detected in the symbiosis of *G. mosseae* with two different plants, but with *G. intraradices* only in very old interactions (104). Also RNA accumulation levels depend on the type of isolate that is used, as for example in the case of genes for chitinase and beta-1,3-glucanase in soybean (105), for two sucrose synthases in maize (106), and for phosphate transporters, as mentioned above (78). Differential expression on the fungal side was detected for two beta-tubulin genes, which were regulated in the opposite direction during development when comparing *G. rosea* with two *Glomus* species (38). The analysis of cDNA arrays presents a tool to look at the expression of much more genes at the same time. Such a screening has been carried out for comparing *G. mosseae*, *G. intraradices*, and *G. rosea* in symbiosis with *M. truncatula* and the number of genes induced in all three types of interactions was surprisingly low (Grunwald, Franken et al., unpublished). Further functional analyses of these genes are necessary to understand the meaning of these differences for the variation of the influence among AM fungal isolates on plant nutrition and plant health.

10.4 BIODIVERSITY IN ECOSYSTEMS

AM fungal abundance and biodiversity can be analyzed by different strategies, for example, using classical approaches such as trap cultures (107) or recently also anastomosis formation (108). Alternative methods are based on isozyme patterns (109) or on DNA as PCR-RFLP (110), SSCP (111), RAPD (112), or AFLP (113). Using these techniques, alone or in combination, revealed that despite the low specificity of AM interactions plant species influence, on the one hand, AM fungal communities in their surrounding soil (114–119). For this reason a certain level of selectivity in the plant–fungus species combination has been proposed (20). This explains, on the other hand, that plant community structure depends on AM fungal diversity, at least in short- and medium-term studies under controlled conditions (120–124). The ecological mechanisms involved are thought to be competition (125) and alteration of resource distribution (126). This mutual dependence implies that reducing the number of plant species when going from natural to agricultural ecosystems would decrease fungal biodiversity. Indeed a comparison of AMF sequences showed a much higher variation in a woodland than in an arable site (127). In order to make such an analysis in a similar soil type, an agriculturally used enclosure in a semi-natural upland grassland was analyzed, showing a reduction in AM fungal variation (128). In contrast, Jansa et al. (27,129) have shown, using molecular and morphological techniques, that AMF diversity is much higher in agricultural systems than previously thought and that certain management practices, such as intensive tillage, can alter the AM fungal community structure. In that study the number of different crop plant species was only three. In a more broad survey of different sites, increased land use intensity was also correlated with a decrease in AMF species richness (130). However, it has been shown that this is not only due to the number of different plant species, but also to the management practices. A cropping system consisting of a native pasture without any fertilization or other plant or soil treatments had the highest numbers of spores and the highest species richness compared to more invasive culturing methods (131). One specific reason is probably the treatment with fertilizers as a reduction in species richness was observed along an anthropogenic nitrogen deposition gradient (132). Another explanation could be the physical breaking of the soil structure: species richness decreased when a natural soil was disturbed (133). Revegetation increased the numbers of spores again, but never reached the variation of the former natural ecosystem. A similar result was obtained after fumigation (134). However, a few results have been obtained that contradict these observations. In a 3-year experiment, cultivation of an old meadow changed the AM fungal community, but did not reduce

TABLE 10.1
Variation Among AM Fungi (summarizing genotype, as well as phenotype
variation and changes in biodiversity)

	Between species	Between isolates	Between nuclei
Genotype variation			
Genome size	15–1000 Mb	nd	nd
rRNA genes	+	+	+
Genes encoding			
chitin synthase	+	+	nd
translation elongation factor	+	nd	–
actin	+	nd	+ (in introns)
β-tubulin	+	nd	gene family
glutathione S-transferase	nd	nd	gene family
binding protein	nd	nd	+
Phenotype variation			
Morphology			
spore size, shape, and color	+	–	
presence of BLOs	+	–	
germination	+	nd	
auxillary cells	+	–	
arbuscule type	+	nd	
vesicles	+	–	
Branching promoting factors	+	nd	
Nutrient exchange			
phosphate	+	nd	
nitrogen	+	+	
iron	+	nd	
carbohydrates	+	+	
Stress tolerance			
heavy metals	+	+	
drought	+	+	
root pathogens	+	nd	
Enzymatic parameters			
various enzymes	+	nd	
superoxide dismutase	+	nd	
RNA accumulation			
fungal general pattern	+	nd	
fungal β-tubulins	+	nd	
plant general pattern	+	nd	
plant chitinase	nd	+	
plant glucanase	nd	+	
plant sucrose synthase	+	+	
plant phosphate transporters	+	nd	
Biodiversity in ecosystems			
Influenced by			
plant species	+	+	
soil management	+	+	

+ = differences detected; – = no differences detected; nd = not determined

species richness (135). A comparison of AM fungal community composition and structure in a conventional and two low-input farming systems over 15 years that management practices did not cause many differences among the fungal communities (136).

Summarizing all these published evidences on changes in biodiversity under taxonomic aspects, a general trend (with exceptions) can be seen: By the cultivation of soils for plant production, mainly *Gigaspora* and *Scutellospora* species are lost and more and more certain *Glomus* species dominate the AM fungal community. This is probably due to their different life cycle strategy. *Glomus* species can not only be propagated by spores, but also by residuals of hyphae and mycorrhizal roots, while the other species depend on their large and relative sensitive spores. If those species should be kept in agricultural ecosystems, management practices must be adapted to their conservation (137,138).

10.5 CONCLUSION

As can be seen from this review, the variation of AM fungi is high on all biological levels from the genotype up to their morphology. This variation undergoes changes in most cases when soils are used for agriculture. It is, however, not clear if those species, which disappear, could not also be beneficial for the cultured crops. In order to use arbuscular mycorrhizal fungi in sustainable agriculture, we, therefore, propose the following strategy (fig. 10.1): (1) Isolates have to be obtained under certain environmental conditions that select for specific characteristics. (2) These isolates have to be screened using a bundle of methods from RNA expression analyses of specific indicator genes up to quality assessments of plant products including consumer acceptance and health properties. (3) Isolates with different features should be cultivated under specific conditions to keep their characteristics. This cultivation must undergo a regular quality monitoring based on molecular

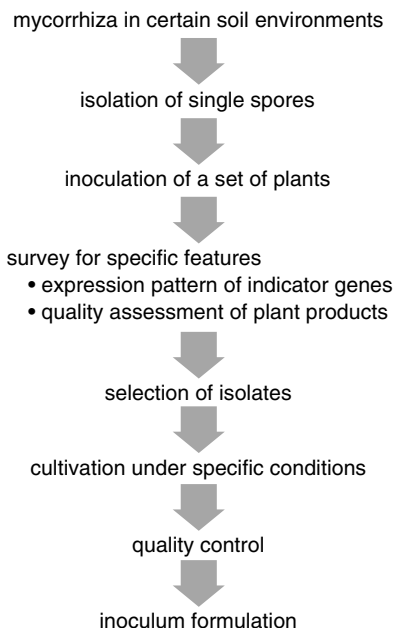


FIGURE 10.1 In order to obtain inocula suitable for certain applications, single spores should be isolated from mycorrhiza under specific environmental conditions and be used for the inoculation of trap cultures. Survey of these cultures for specific features will help to choose a collection of isolates which have to be further cultivated under defined conditions. Quality control will ascertain that the designated characteristics have been successfully conserved. Mixing isolates, which are able to complement one another, will result in an inoculum being design for a specific application purpose.

identification methods. (4) Inocula can be finally formulated as mixtures from isolates for specific demands. (5) Finally, management practices have to be adapted to stabilize the diversity of the inoculum and to keep its specific features over a longer period. Such a strategy needs further intense basic and applied research on both the plant and the fungal site in order not only to correlate, but to connect in a cause-and-effect chain genetic and functional diversity.

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11 Diversity of Protozoa

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Agricultural production systems, like all terrestrial ecosystems, consist of two obligatory mutualistic components, an above-ground producer subsystem and a below-ground decomposer system. Plants are responsible for the amount of carbon entering the system, but the below-ground heterotrophic consumers ultimately govern the availability of nutrients (produced by mineralization of organic matter from above-ground organisms) required for plant productivity (1). Conventional agriculture differs from natural ecosystems by supplying a large part of the nutrients as chemical fertilizers and/or organic residues (2) that nourish above-ground crops and influence below-ground organisms, hence understanding structure and functioning of the below-ground system is necessary to improve crop and soil properties.

Agricultural plant production depends upon decomposition of fertilizers and soil organic matter (much of it in plant and animal residues) by bacteria and fungi, which immobilize the extracted nutrients into their bodies (chapters 7–9 in this volume). The soil fauna graze the microflora and release most of the soluble nutrients through excretions to plant roots through two food chains: bacterial, grazed by protozoa and bacterial feeding nematodes; and fungal, grazed by fungal feeding nematodes and microarthropods (1).

These actions occur mainly in the water films covering soil aggregates and filling their pores, where bacteria and their protozoan and nematode consumers process matter more rapidly than the fungi and their consumers (3). Protozoa have higher turnover rates and exert greater impact mass for mass than other fauna, for example, can be responsible for 20–40% net N recycling (4).

11.1 PROTOZOAN BIODIVERSITY

11.1.1 PROTOZOAN GROUPS

With the exception of ciliates, soil protozoa are a polyphyletic assemblage of unicellular eukaryotes comprising four groups: the rapidly growing heterotrophic flagellates, amoebae, ciliates, and the slower-growing shelled amoebae or testacea. Protozoan distribution is influenced by pore space diameters. Flagellates and small amoebae can occupy all pore sizes down to 6 μm , but larger protozoa are restricted to spaces $\geq 30 \mu\text{m}$, thus there is a protozoan domain of the smaller pore spaces and a protozoan–nematode domain of the larger ones (5). Some common protozoa found in agricultural soils are illustrated in figure 11.1.

Flagellates are typically swimming organisms, but many can extend pseudopodia or employ amoeboid movement to move along surfaces. They appear immediately and abundantly when soils are wetted. Routine identification is virtually impossible because most species require EM techniques and pure cultures for identification (6), hence most soil flagellate studies report numbers only.

Amoebae adhere close to substrates, can move in thin water films, and their plasticity enables them to invade small crevices and all pore space forms. Many species can extend pseudopodia into tiny pore spaces to engulf bacteria (7). The approximately 60 species of soil amoebae can be grouped into four morphotypes for ecological studies: type 1 with finger-like or tapering pseudopodia (e.g., *Acanthamoeba*, *Mayorella*); type 2, noneruptive locomoting limax (e.g., *Hartmanella*, *Saccamoeba*); type 3, eruptive locomoting limax (Vahlkampfiidae); and type 4, discoid or flattened (e.g., *Platyamoeba*, *Vanella*) (8).

The less numerous but more diverse and larger ciliates and testacea are restricted to the larger pore spaces, where they are subjected to greater environmental stress, hence both groups exhibit a wide spectrum of r/K selection (9) and can serve as bioindicators of soil conditions. Ciliates are the most complex protozoa, distinguished by cilia, a cytostome (cell mouth), and well-defined bodies. Ciliates can be grouped into three taxocenes, pioneer r-selected Colpodida plus two hypotrich genera, competitive K-selected Polyhymenophora, and intermediate remaining taxa. Dividing the number of species in the first group by the number of species in the second produces a C/P ratio, where $C/P > 1.00$ indicates a stressed soil of low productivity, and $C/P < 1.00$ a more stable, productive soil (9, 10).

The shell of testaceans conveys information about moisture fluctuations: the shape changes from large vaulted in moist habitats to smaller vaulted, hemispherical, and wedge-shaped in drier soils. Of the approximately 300 terrestrial species, a few small species of the genera *Cryptodiffugia*, *Euglypha*, *Trinema*, and *Phryganella* are r-selected pioneers (10) predominating in agricultural and arid soils (11,12). Larger testacea with lobose pseudopodia and hemispherical and vaulted shells increase the species diversity in more stable soils (e.g., grasslands and forests).

Environmental changes in soil can be rapid and severe, and protozoa survive drying/wetting, temperature, translocation by animals, and other stresses by forming resistant cysts. Most soil protozoa remain dormant for long periods of time, making estimates of actively metabolizing protozoa difficult. The concept of “growth potential” (6, 13), discussed below, builds on this complication.

11.1.2 METHODS OF STUDY

The small size and close association of protozoa with soil particles prevents extraction in most situations; hence culturing methods are usually required for enumeration and isolation of species. In arable soils, organisms can be separated from soil particles by density centrifugation followed by staining, allowing observation of metabolically active protozoa (14). Direct counts can be made of active ciliates by examining soil suspensions drop-by-drop until at least 0.4 g of fresh soil has been examined, and if a stain is added to a second fresh sample, shell-bearing testacea can also be counted (15). Mounting small soil samples on slides followed by staining provides a permanent record of testacea (16, 17).

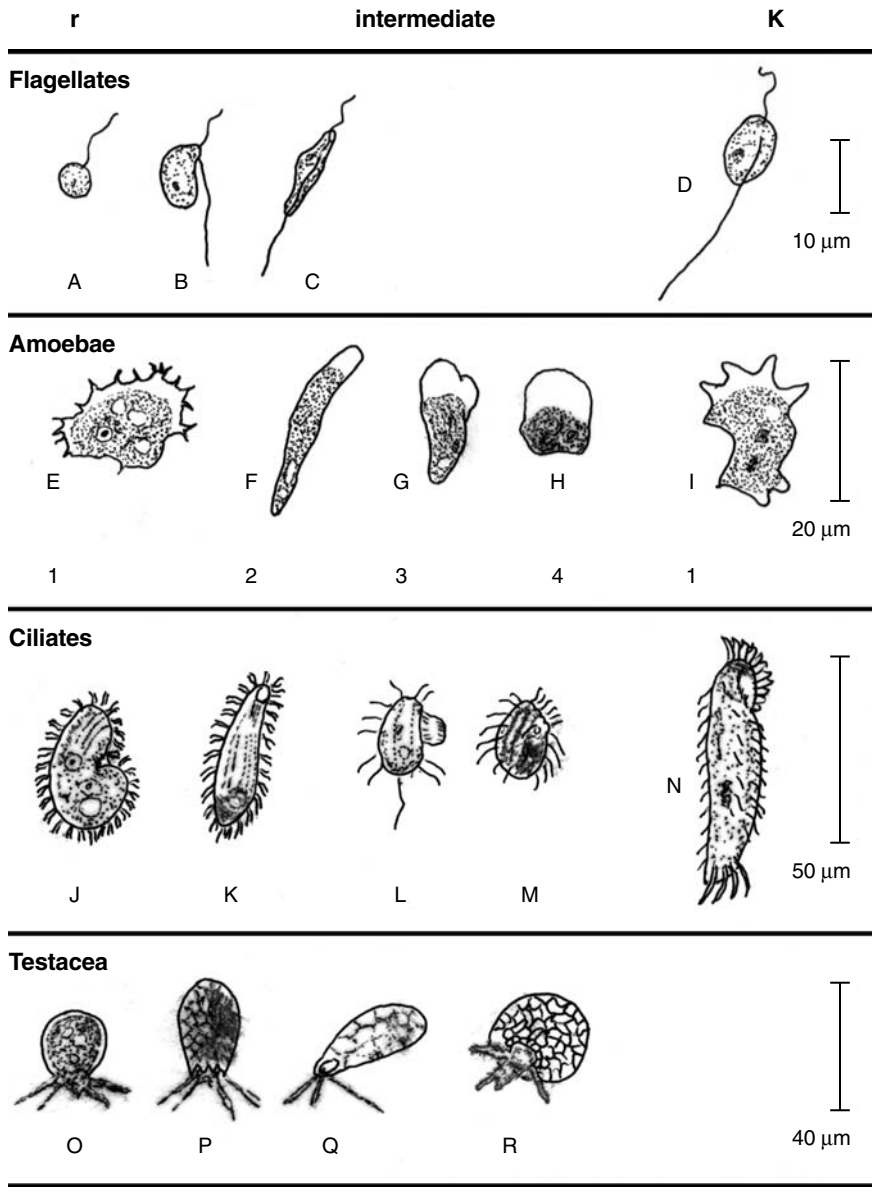


FIGURE 11.1 Some common protozoa in agroecosystems. r-selected species are arranged on the left and K-selected species on the right. Numbers 1–4 identify the four morphotypes of amoebae. (A) *Oikomonas*, (B) *Bodo*, (C) *Cercomonas*, (D) *Anisonema*, (E) *Acanthamoeba*, (F) *Hartmanella*, (G) *Vahlkampfid*, (H) *Platyamoeba*, (I) *Mayorella*, (J) *Colpoda*, (K) *Platyophrya*, (L) *Cyclidium*, (M) *Leptopharynx*, (N) *Holosticha*, (O) *Cryptodiffugia*, (P) *Euglypha*, (Q) *Trinema*, and (R) *Phryganella*.

The most widely used culture method for naked protozoa is the Darbyshire et al. (18) modification of the most probable number (MPN) dilution technique. Pretreatment of a second sample by heating, desiccation, or freezing destroys living forms, the difference between the two samples representing the number of cysts. A second culture method for amoebae (8) and ciliates (19) involves dilution of a sample and small aliquots of it (each containing a single individual) into microtiter plates, and counting the number of positive wells after incubation. This culture method, combined with direct counts of fresh soil, provides a nondestructive method to discern proportions of active

and encysted ciliates (19). Finlay et al. (13) have introduced a method of examining quantitatively wetted soil samples at specific time intervals to estimate the “growth potential of the soil protozoan community.” This concept recognizes that a large fraction of the protozoan population is encysted, so that total numbers (active + cysts) should be considered a potential for grazing (6, 13), and can be applied to understanding soil bacterial activity (4). Despite difficulties of enumeration (especially by the MPN method), protozoan numbers have been multiplied by conversion factors for biomass, biovolume, and metabolic activity (bacterial consumption) (6) to provide information to assess protozoan activity in soils and construct models of soil food webs and nutrient recycling (see chapter 19 in this volume).

Species richness must also be determined by culture procedures, the best being the nonflooded Petri dish method (15), where 10–50 g of sample is placed in a Petri dish and saturated, but not flooded, with water, and examined at 3 to 4-day intervals for a month. Most amoebae will be found by streaking samples of soil onto bacterized non-nutrient agar plates and examining 4–5 days later. Many (especially large) testacea are found in the Petri dish method, but additional smaller species will be found on slides containing stained soil samples (16, 17).

11.1.3 PROTOZOA AS BIOINDICATORS

Protozoa are increasingly used as bioindicators of soil conditions because of their delicate cell membranes, rapid growth, and restricted movements in soil; and they can react more quickly to environmental changes than higher fauna. The wide ranges of morphologies of ciliates and testacea, and their restriction to the larger pore spaces (subjected to greater environmental stresses), provide a multispecies approach enabling community analyses to describe soil conditions. The abundance of flagellates and amoebae in a soil is a better index of bacterial activity than bacterial numbers or biomass because these two groups of protozoa conduct most nutrient mineralization in water films (20). The use of protozoa has been restricted by difficulties in enumeration, time needed for identification, and lack of easy-to-use taxonomic literature (21), but examples of their application are presented in the discussion of controls of biodiversity in Section 3.

11.1.4 PROTOZOAN BIODIVERSITY IN AGRICULTURAL PRODUCTION SYSTEMS

Soil protozoan biodiversity can best be understood by relating numbers and species to the structure and conditions of their three-dimensional mosaic of soil aggregates and pore spaces, containing microsites or “hot spots” of organic matter and their accompanying microorganisms and soil fauna, scattered discontinuously. Additional hot spots include the rhizosphere around plant roots and casts and burrows of earthworms. The soil mosaic is frequently rearranged by growth of plant roots and the movements of macrofauna—ants, termites, and especially earthworms. Bacteria and their protozoan predators are restricted within water films, so migration between hot spots is determined by the extent of connecting water films (22), and translocation by plant root growth and animal activities. Soil protozoan biodiversity describes a population of spatially separated individuals waiting for the opportunity to exploit their hot spots, more than niche overlap or functional redundancy. Well-managed agricultural soils provide favorable conditions for diverse protozoan populations.

Recorded numbers of soil flagellates and amoebae in arable lands estimated by the MPN technique are between 10,000 and 100,000 per gram of soil (6). Ciliates and testacea average about an order of magnitude lower (6, 13). Under favorable conditions of rainfall or supply of organic matter, the number of flagellates and amoebae may increase 6–20 times in a few days (23,24), flagellates responding more quickly than amoebae. Such sudden changes demonstrate that initial numbers of these two groups should be considered as estimates of the growth potential of the soil protozoan community (6, 13), a measure of bacterial activity. The universal genus *Acanthamoeba*

is almost always present, and type 3 vahlkampfid amoebae are often found under conditions of moisture and recent rainfall (25) (Bamforth, unpublished).

The less numerous “bioindicator” ciliates and testacea can be counted by direct (15) or aliquot (19) methods. Ciliates in arable soils usually range between 200 and 800 per gram of soil (19). Under nonstressed conditions, active individuals comprise 40–60% of a total population; a few r-selected colpodids furnish about 50%, and about a dozen other intermediate species furnish most of the remaining individuals in both active and total numbers in a soil ciliate population. The “stop-and-start” conditions produced by temperature and moisture fluctuations favor the r-selected colpodids, with their broad tolerance to abiotic extremes, rapid encystment/excystment abilities, and rapid multiplication (19). Most of the biodiversity of soil ciliate populations, which range from 30 to 50 species, is produced by a small proportion of the individuals (11,15, 19, 26). Among these rare species are many K-selected Polyhmenophora, which can be compared to the number of colpodid species in the soil to produce the C/P ratio. Ciliate species richness is only slightly decreased in arable lands compared to neighboring natural systems (19, 27), but testacea are reduced to the few euroecious species mentioned earlier, a small residue of the diverse communities found in the former natural habitats (27). Some tilled soils also have a low ciliate biodiversity (26), which, with the impoverished testacean community, warn of possible land degradation, leading to desertification.

11.2 STRUCTURE AND FUNCTIONAL RELATIONSHIPS

Soil protozoa graze bacteria, and to a lesser extent fungi, especially in the rhizosphere of plant roots. Protozoa compete with and are preyed upon by nematodes in water films; and are ingested by and are moved through soil by earthworms.

11.2.1 BACTERIA AND FUNGI

Most soil flagellates, amoebae, and common (usually small) ciliates feed on the bacteria in their water films. Smaller testacea likewise feed on bacteria, but many species also feed on humus particles (28). Fungi grow out of and in between water films, but portions of their mycelia and spores in water films can be attacked by a number of taxonomically distant amoebae, but most of these also feed on bacteria (6) One family of colpodid ciliates, the Grossglockneridae, are obligately mycophagous, and possess a specialized oral apparatus for piercing yeast cells (29). A few testacea, for example, *Phryganella*, can attack fungal hyphae (30). Mycophagy is being observed more frequently in soil protozoa, but its importance is not understood. Several hundred species contribute individuals to the numerically small but species-rich portion of soil ciliate populations, and about 50% of these species are predaceous. Most of the others are omnivorous, with wide nutritional niches but tending to feed at the lower level of the food chain (i.e., bacteria).

Most soil protozoa consume bacteria and excrete up to 60% of their ingested nutrients, thus enhancing the turnover and mineralization of organic carbon and nitrogen (31). Flagellates and small amoebae eat bacteria in small pore spaces inaccessible to ciliates and nematodes, thus furnish most of the protozoa. Microcosm studies show that amoebae appear to be responsible for 20–40% of net N mineralization in field studies (4), and are also responsible for mineralization of P (32) and S (33), although the mechanisms of the two latter processes are not understood. Protozoan grazing keeps bacterial populations at a lower level (preventing them from becoming limited by density-dependent factors) and furnishes nutrients to bacteria (the “microbial loop”) as well as to plants, resulting in increasing bacterial activity and decomposition. Excretions of higher fauna also add some nutrients into the soil, but protozoan–bacterial interactions are central to soil nutrient cycles because they act as catalysts of these cycles (31).

11.2.2 INTERACTION WITH PLANTS: THE RHIZOSPHERE

Some of the photosynthetically fixed carbon of plants is released in root exudates, stimulating bacteria and their predatory protozoa in the root zone or rhizosphere. Protozoa enhance plant growth by mineralizing more N (from the large bacterial population), and secreting plant-like hormonal active substances, although the mechanisms are poorly understood (34, 35).

11.2.3 NEMATODES

Bactivorious nematodes compete with protozoa, but like ciliates and testacea, are restricted to the larger pore spaces. Protozoa reproduce more rapidly than nematodes and can be up to 10 times more efficient than nematodes in yield (36). However, some newly created hot spots, such as a rapidly growing rhizosphere zone and freshly buried plant residues, can support a large population of nematodes because their migration ability gives some competitive advantage over the relatively static protozoa. Whether protozoa or nematodes are dominant at an active site may depend on substrate and conditions (37). Protozoa (and bactivorious nematodes) also furnish food for predatory nematodes, which in turn are preyed upon by microarthropods.

11.2.4 EARTHWORMS

Protozoa are part of the soil particles ingested by earthworms.. Active protozoa are digested and supply important dietary elements to the worm but many encysted protozoa survive passage through the earthworm gut, and upon egestion, feed on the increased numbers of bacteria and fungi found in worm casts (38). Earthworms, through their burrowing activities, aid bacteria and their protozoan predators by increasing soil porosity and soil aggregates, and disperse protozoa attached to their bodies and passage through their guts to new positions in the soil. Earthworms line their burrows with protein-rich mucous, providing very favorable environments for bacteria and their protozoan predators (39). Earthworms and protozoa often constitute the largest biomass of soil fauna (15) A soil rich in earthworms is rich in protozoa.

11.3 FACTORS CONTROLLING BIODIVERSITY

Agricultural production systems can be considered modified grasslands, characterized by lack of plant canopies and litters that protect soils, and modified by intensive application of artificial fertilizers and biocides, and use of farm machinery to increase crop production. Sustainable approaches seek to minimize topsoil disturbance, reduce inputs, and substitute organic for mineral fertilizers in order to stimulate the below-ground microflora and fauna that supply plants with nutrients.

11.3.1 CULTIVATION MANAGEMENT

In conventional agriculture, plowing incorporates crop residues into the soil profile to produce homogenous soils resembling an early stage in ecological succession, thus favoring the bacterial food chain (i.e., protozoa and bactivorious nematodes). Minimal tillage or ecofarming leaves crop residues on the surface where a top-rich organic layer develops, enhancing the fungal food chain: fungivorous nematodes, Collembola, and earthworms (40, 41). This layer also supports a large bacterial community, producing a greater flagellate, amoebae, and testacean biomass (27, 42,43). However, ciliates do not show any great differences between the two systems. Crop yield is similar in both systems, but ecofarming has lower operating expenses, and ecologically shows that biological processes compensate by reducing fertilizers and pesticides. Ecofarming is more resilient during poor climate periods and in semiarid regions, and conserves the below-ground biodiversity needed for long-term soil sustainability (41,44).

11.3.2 FERTILIZERS

Agricultural management often increases crop yields by adding fertilizers to soils, and these additions usually enhance bacterial and protozoan populations. Mineral fertilizers sometimes slightly depress and alter the community structure of soil protozoa, but the effects are not permanent (15). Organic residues—sewage sludges, animal manures, and plant residues—are less costly, and contain organic matter and microorganisms, thus are more similar to the natural organic matter in soils. Bacteria and their accompanying protozoa, especially amoebae, increase quickly and achieve greater biomass than nematodes, due to more rapid reproduction and ability to inhabit smaller soil pores. Most soil fauna, especially earthworms, whose activities enhance protozoan biodiversity, also increase (45).

11.3.3 BIOCIDES

Biocides applied to control insects, weeds, and pathogenic fungi can also stress soil protozoa, whose susceptibility is similar to other soil animals. Biocides and their degradation intermediates can bind to and become incorporated into soil organic matter, and influence the humus-associated testacea. Herbicides have little direct effect on protozoa, but may alter activities of soil bacteria, either directly by altering bacterial metabolism or indirectly by eliminating vegetation that supply organic matter for bacterial action (and protozoan predation). Insecticides are more toxic and reduce soil protozoan populations, which may not recover for 60–90 days after application (11,46). Fungicides have varied effects (e.g., fenpropimorph) causes a sudden decrease in both bacteria and protozoan populations, followed by rapid recovery and increase (47, 48), whereas others (e.g., mancozeb) exert little effect (11).

11.3.4 SOIL COMPACTION

The use of heavy machinery in modern farming compacts soils, destroying worm and plant root channels and reducing soil porosity, thus limiting bacteria–protozoan activity (49, 50). Compaction can also produce anaerobic conditions that inhibit protozoa and reduce the metabolism of their bacterial prey (51).

11.3.5 SOIL RESTORATION AND CONSERVATION

Exhausted agricultural and other damaged soils must acquire an interacting microflora and fauna in order to be restored for productive farming. Propagules of bacteria, fungi, and protozoa arrive by air, water, and animals, and colonize in a specific succession: for protozoa, the sequence is flagellates, small amoebae, the ciliate genus *Colpoda*, and small testacea. Many of these r-selected taxa remain, but intermediate and K-selected species arrive to increase species richness and numbers, indicated by the $C/P > 1$ ciliate species ratio in early weeks decreasing to $C/P < 1$ later, indicating a mature population (10). Addition of organic fertilizers enhances colonization (9).

11.3.6 SUMMARY

Agricultural production systems depend on the underground decomposers and fauna to recycle nutrients to sustain crop plants. Much of this mineralization is accomplished in the water films by a diversity of protozoa and bacterivorous nematodes, which also interact with plant roots in the rhizosphere and with earthworms. Ecofarming creates a more favorable environment for the soil biota. Threats to the biodiversity of all soil biota include excessive use of mineral fertilizers and biocides and soil compaction.

Rapid multiplication and close association with soil particles make protozoa excellent indicators of soil conditions, and can be used to monitor agricultural soil development and conditions, and effects of disturbance (e.g., fertilizers, biocides, and compaction).

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12 Diversity of Nematodes

Gregor W. Yeates

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12.1 INTRODUCTION

Plant and soil nematodes may be both abundant (>10 million m^{-2}) and diverse (> 90 species) in a particular agricultural production system. Although for centuries considered as the plant pathogens they are now, through their effects in increasing the availability of plant nutrients, also known to make positive contributions to ecosystem processes. The plant-pathogenic aspects of nematodes in temperate, subtropical, and tropical agricultural systems are reviewed in Evans et al. (1993) and Luc et al. (1990). Some nematodes have alternate life-cycles with a bacterial- or fungal-feeding life-cycle contrasting with one parasitic in invertebrates; such species may play important roles in regulating populations of pest invertebrates in agricultural production systems. Nematode parasites of grazing animals also occur in agricultural systems but, although some (e.g., Trichostrongylidae) have bacterial-feeding juveniles in soil and dung, they are not included in this chapter, which focuses on nematodes of cropping, horticultural, and pastoral systems.

This chapter (1) outlines the systematic, morphological, and biological diversity of nematodes in agricultural production systems, (2) describes nematode feeding types and how their feeding contributes to processes of production and decomposition, and (3) gives an account of the impact of management practices on the abundance and diversity of nematode assemblages.

12.2 NEMATODE DIVERSITY

The basic nematode form is essentially a tube forming the gut lying within a tube forming the body wall, and the reproductive organs in the body cavity. It is a template for modification with marked adaptation of the head region for utilizing a range of food resources (fig. 12.1). Adult body sizes range from 0.3 mm to 8.4 m in length. Many nematodes show stage-specific activity among the egg, four juvenile stages, and adults. Stages may be resistant, infective, or apparently missing; there may be moults before hatching. Different stages of a single species may differ in size, morphology,

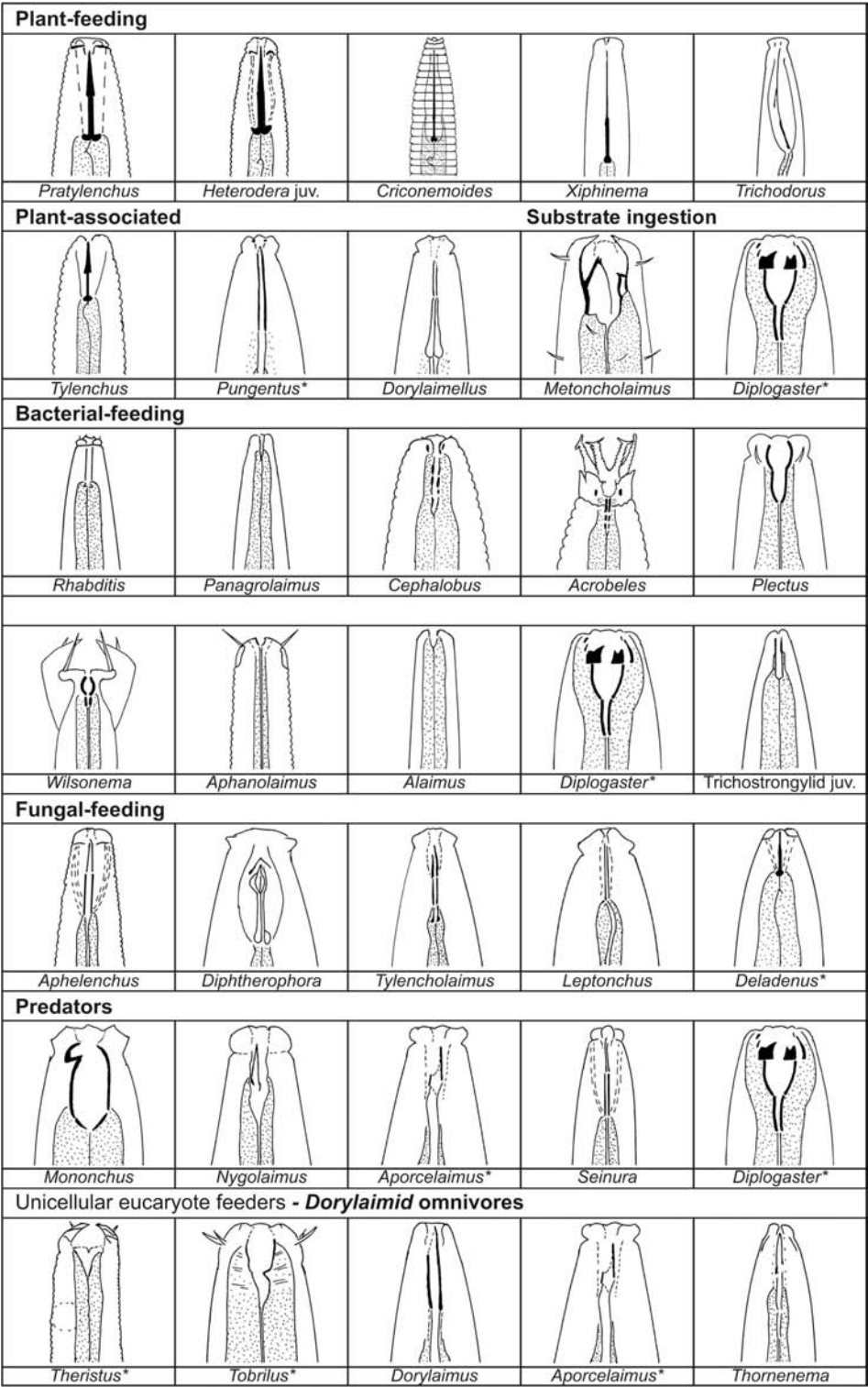


FIGURE 12.1 Head structures of soil-inhabiting stages of selected nematode genera to illustrate the range of structures associated with each of the eight feeding types. Genera which may be allocated to more than one feeding group are indicated by * and may be shown under each type. Females illustrated except for *Heterodera* and trichostrongylids where juveniles are shown. Drawn at various scales.

and feeding habits and, in some groups, there are separate “free-living” and “parasitic” life-cycles. The phylum Nematoda is species rich, and nematodes are the most abundant multicellular animals on earth (Yeates 2004).

12.2.1 FOOD, ENERGY, AND FEEDING TYPE CLASSIFICATION

Nematodes are heterotrophic and rely on energy fixed by other organisms in primary production. The food also supplies necessary materials (e.g., carbon, nitrogen, phosphorus), and study of the fluxes of these is central to understanding the biology of nematodes and their role in agricultural production systems. Nematodes directly using living autotrophs are “grazers” and belong to “grazing foodwebs.” whereas those based on nonliving organic matter belong to “detritus foodweb” (Petersen and Luxton 1982). The materials that heterotrophic organisms utilize can flow in closed cycles, and it is these fluxes (especially those of C, N, and P) that generally are the prime focus of system models and “budget” studies of animals.

In striving to understand relationships between nematode head and stoma morphology and feeding habits, many feeding type classifications have been proposed; Yeates et al. (1993a) and Moens et al. (2004) give reviews. The diversity of nematode head structures has been illustrated by Zunke and Perry (1997) and by other authors. For this chapter the following eight feeding groups, typical head structures of which are illustrated in figure 12.1, are relevant: (1) *Plant-feeding* nematodes use higher plant tissue and fluids as their food resource. They have a tylenchid stomatostylet, a dorylaimid odontostyle, or a triplonchid onchiostyle. A range of subgroups may be distinguished, with the food resource of the delicate-speared Tylenchidae and Psilenchidae still being unresolved. As high population densities of these families are often found in the rhizosphere, apparently without any adverse effects on plants, they may be categorized as (2) *plant-associated* nematodes (Yeates et al. 1993b; Yeates 1999); recent studies have shown that some can maintain populations as fungal-feeders (Okada et al. 2002). (3) *Fungal feeding* nematodes use a stomatostylet, odontostyle, or onchiostyle in feeding. There are both obligate hyphal feeders and the alternate life cycles of some parasites of invertebrates. (4) *Bacterial-feeding* nematodes generally ingest bacterial cells whole. There is evidence both for crushing of bacterial cells (Bird and Ryder 1993) and for bacteria surviving passage through the intestine (Yeates 1970). In some, particularly those with a broad stoma (e.g., Diplogasteridae, Mononchidae), food in addition to bacteria may be utilized; predation on amoebae or nematodes has been documented (e.g., Diplogasteridae, Mononchidae) (Elliott et al. 1985; Allen-Morley and Coleman 1989; Yeates 1969). (5) *Substrate ingestion* represents a feeding activity where ingestion of the substrate itself appears to be the purpose. *Diploenteron colobocercus* may ingest agar covered with a bacterial lawn (Yeates 1970). It is unclear whether the substrate and/or the microbial growth are utilized. (6) Many nematodes are *predators*, feeding on a variety of invertebrate metazoa. Adaptations of the stoma include teeth, stylet, and mandibles, although ingestion of prey by nematodes with unarmed stomas has been observed. Depending largely on the nature of the stomal armature, prey body contents may be sucked in, or ingested whole (Small 1987). Predation is often regarded as the principal feeding strategy (e.g., in *Seinura*, *Nygolaimus*, *Labronema*), but that *Mononchus*, *Diploenteron*, and other “predators” can be successfully cultured on bacteria (Allen-Morley and Coleman 1989; Yeates 1987a) suggests these nematodes may be facultative predators. (7) *Unicellular eucaryote feeders* utilize diatoms, other microalgae, yeasts, fungal spores, and protozoa. Cells may be pierced or ingested whole. (8) *Free-living stages of animal parasites* may contribute to soil processes and should then be included under the appropriate category (bacterial- or fungal-feeding). If ensheathed or otherwise inactive they should be treated separately.

In nematology the term “omnivore” has usually been applied to some dorylaimids to indicate a “mixed” diet but it is a misnomer as in ecological terms an omnivore is an organism that feeds at more than one trophic level. Thus, bacterial-feeding nematodes that also feed on protozoa, and predators feeding on nematode prey as well as on bacteria, are in fact omnivores. The only single-source feeders may be plant and hyphal feeders. Also, during development many nematodes shift

from feeding on one trophic level to another; these are “life-history omnivores” (Yeates 1987a). Several ecological studies have shown populations of both omnivores and predators to respond similarly to various food-web parameters and they may represent a single functional group. Existing knowledge is such that “dorylaimid omnivores” are retained as a feeding group in this chapter. Nematode feeding types/groups are resource guilds. They are not necessarily functional groups, as this would imply that they share both food and predators.

12.2.2 EFFECTS OF NEMATODE FEEDING

Plant-feeding nematodes adversely affect economic yield when they stress plants, and many examples have been documented (fig. 12.2). Assessment of preplant populations of nematodes such as *Pratylenchus*, *Belonolaimus*, and *Xiphinema* and juveniles of *Meloidogyne*, *Globodera*, and *Heterodera* not only illustrates how nematodes can be indicators of soil conditions but also forms the basis for modeling both nematode damage to crop damage and nematode population changes (McSorley and Phillips 1993). Many stylet-bearing nematodes in the rhizosphere have, however, not been demonstrated to have an adverse effect on plant growth. Large populations of *Tylenchus*, *Filenchus*, *Cephalenchus*, *Boleodorus* and other Tylenchidae, as well as other genera such as *Paratylenchus*, can occur in the rhizosphere of apparently healthy crops. At low densities *Rotylenchus uniformis* and *Heterodera trifolii* appear to stimulate growth of carrots and white clover, respectively (Seinhorst and Kozłowska 1977; Yeates 1978).

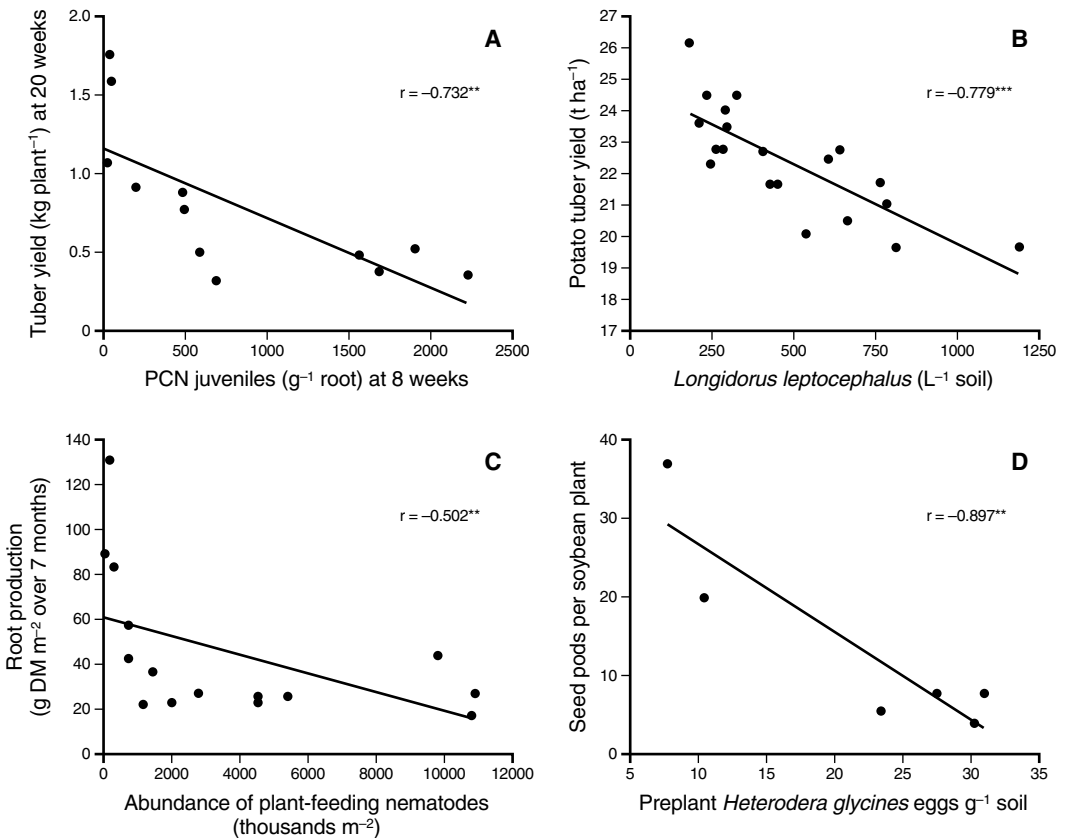


FIGURE 12.2 Negative, pathogenic relationships between the abundance of plant-feeding nematode populations and crop growth or yield. Graphs A–D have been derived from Trudgill et al. (1975), Sykes (1979), Wasilewska (1999), and Yamada et al. (2003), respectively.

Nematodes may affect nutrient mineralization both directly, from their excretion of ingested nutrients that are not used in tissue production, and indirectly, from modification of the microbial community, accelerated turnover of microbial cells, inoculation of new substrates with microorganisms, and leakage into the rhizosphere of nutrients from feeding sites (Griffiths and Bardgett 1997). In a classic paper, Ingham et al. (1985) demonstrated that experimental systems containing *Pelodera* (Rhabditidae) or *Acrobeloides* (Cephalobidae) had higher bacterial densities than similar microcosms without nematodes. Plants growing in such microcosms grew faster and initially took up more nitrogen, because of increased nitrogen mineralization by bacteria, $\text{NH}_4^+\text{-N}$ excretion by nematodes, and greater initial exploitation of soil by plant roots (table 12.1). The positive contribution of fungal-feeding nematodes to nutrient mineralization was best demonstrated when substrates with a C:N ratio of ≥ 15 were used (table 12.2). There was a significant increase in nitrogen mineralization when fungi were grazed by *Aphelenchus* or *Aphelenchoides* (Aphelenchida) (Chen and Ferris 1999, 2000). This is in keeping with ecological situations in which fungi are known to make a significant contribution to soil processes.

The use of an isotopic labeling technique demonstrated that species of *Heterodera*, *Xiphinema*, *Pratylenchus*, and *Meloidogyne* increase leakage of photosynthate from roots and stimulate biological

TABLE 12.1
Effect of Increasing Microfloral and Microfaunal Diversity (i.e., food-web complexity) on Soil N and Growth of a Grass (*Bouteloua gracilis*) in Microcosms

Organisms present	$\text{NH}_4^+\text{-N}$ ($\mu\text{g/g}$ soil)	Shoot biomass (mg)
Plant	33.0	2.1
plant + bacteria	32.2	2.6
plant + bacteria + bacterial-feeding nematode	45.5	6.6
plant + fungus	48.6	7.5
plant + fungus + fungal-feeding nematode	49.1	8.6
plant +bacteria + fungus + b/f nematode + f/f nematode	46.4	13.5
HSD ($p < 0.05$)	10.2	3.0

Derived from Ingham et al. (1985)

TABLE 12.2
Substrate C:N Ratios at Which Six Nematodes Significantly ($p < 0.05$) Increased N-Mineralization in Experimental Columns (*E. coli* and *Rhizoctonia solani* were food sources)

Substrate C:N	Bacterial-feeding nematodes				Fungal-feeding nematodes	
	<i>Acrobeloides</i> <i>buetschlii</i>	<i>Bursilla</i> <i>labiata</i>	<i>Cephalobus</i> <i>persegnis</i>	<i>Cruzinema</i> <i>tripartitum</i>	<i>Aphelenchus</i> <i>avenae</i>	<i>Aphelenchoides</i> <i>composticola</i>
11	+	+	+	+	n.s. ^a	n.s.
15	+	+	+	+	+	n.s.
20	+	+	+	n.s.	+	+
30	+	+	+	n.s.	+	+
40	+	+	+	n.s.	+	+

^a not significant ($p > 0.05$)

Derived from Ferris et al. (1998), Chen and Ferris (1999, 2000)

TABLE 12.3
Increase in Percentage of ¹⁴C in Soil Microbial
Biomass in the Presence of Nematodes, 15 Days After
Pulse-Labeling Pots of White Clover (*Trifolium repens*)

Nematode	¹⁴ C in soil microbial biomass
(control)	1.17
<i>Heterodera trifolii</i>	1.57
<i>Meloidogyne hapla</i>	1.72
<i>Meloidogyne trifoliophila</i>	1.47
<i>Xiphinema diversicaudatum</i>	2.45
<i>Pratylenchus</i> sp.	2.50
LSD (0.05)	0.49
Derived from Yeates et al. (1999a)	

activity in the rhizosphere (Yeates et al. 1999a; Denton et al. 1999) (table 12.3). Jenkinson (1990) and Anderson and Domsch (1990) quantified nutrients in soil microbial pools in soils and, given that microbial processes in the rhizosphere play key roles in making nutrients available for plant uptake, this transfer of recently photosynthesized carbon to the soil microbial biomass essentially closes the loop between root-feeding nematodes, microbial-feeding nematodes, and the overall positive relation between nematodes and plant growth observed in a range of situations (fig 12.3). The contributions of soil-inhabiting nematodes to the processes outlined in table 12.1, table 12.2, and table 12.3 provide a strong correlative basis for inferring that the size and diversity of nematode populations contribute to ecosystem productivity. The problems in unraveling the complex relationships between diversity and productivity, and demonstrating causality, have been addressed experimentally by, for example, Bardgett and Chan (1999), Wardle et al. (2003a, 2003b, 2004), and reviewed by Wolters (2001) and Wardle (2002). Much remains to be understood. Examination of differences in C:N:P ratios between trophic levels, with “excess” nutrients being excreted in addition to normal metabolic costs, may prove a fruitful area of study.

12.2.3 NEMATODES IN BIOLOGICAL CONTROL OF AGRICULTURAL PESTS

Use of entomopathogenic nematodes in biological control in agroecosystems provides direct evidence of a positive feedback of nematode activity. Entomopathogenic nematodes such as *Steinernema* and *Heterorhabditis* (Rhabditina) are applied to manage insect pests of cranberries, turf-grass, artichokes, mushrooms, ornamentals, and many other pests in horticultural, agricultural, and domestic situations (Gaugler 2002). This effect is not due to nematodes alone. The pathogenicity depends on a mutualistic nematode–bacterial complex (with strains of *Xenorhabdus* and *Photorhabdus*, respectively). Slugs in agroecosystems can also be managed using the nematode *Phasmarhabditis hermaphrodita* and associated bacteria (Wilson et al. 1995).

12.3 DIVERSE NEMATODES OF EACH FEEDING GROUP COEXIST

In most soils there is a range of morphologically distinct nematode species in each feeding group. Understanding the relationship between such taxa is a problem throughout soil biology (e.g., Giller et al. 1997; Griffiths et al. 2001; Maraun et al. 2003; Wardle et al. 2003a, 2003b). Conventional wisdom is that those species differ in their use of resources in time or in space. This section serves to indicate ways in which nematodes with broadly similar feeding habits may coexist in a soil.

Host specificity (e.g., *Heterodera avenae* on wheat cf. *H. trifolii* on white clover), site differentiation (e.g., *Anguina tritici* in wheat florets cf. *Heterodera avenae* in wheat roots), and temporal

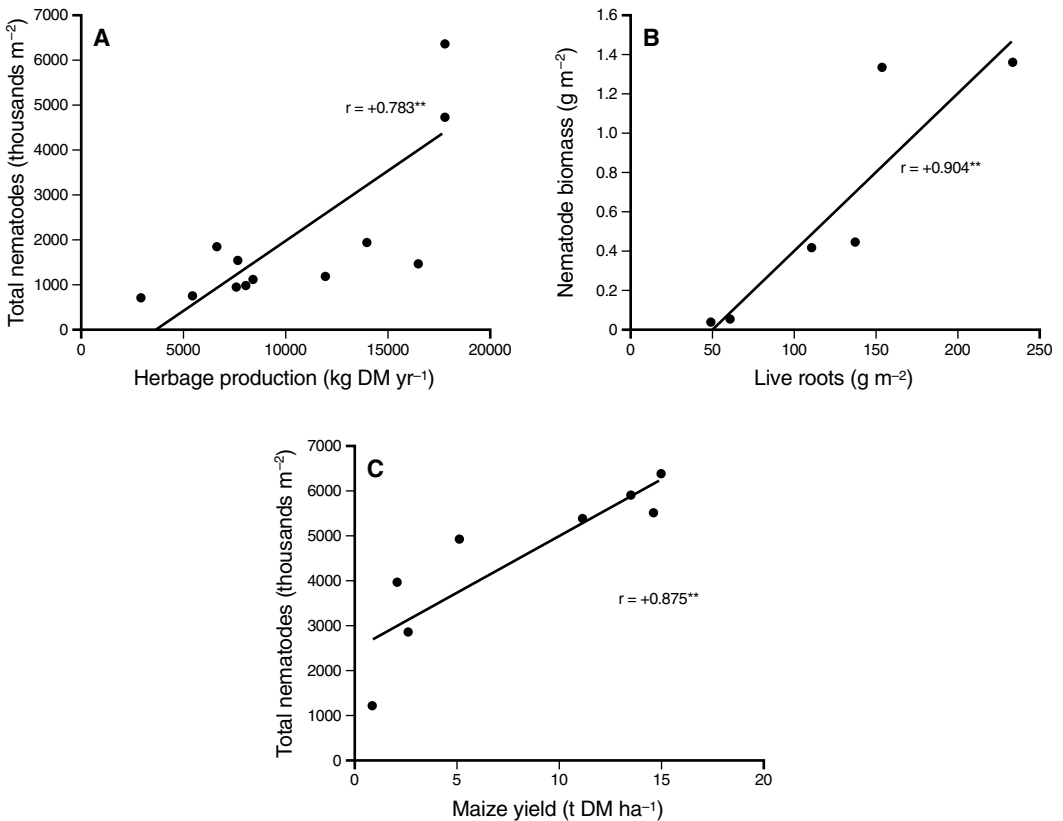


FIGURE 12.3 Positive relationships between total nematode abundance or biomass and plant growth or yield. Graphs A–C have been derived from Yeates (1984), French (1979), and Korthals et al. (1996), respectively.

separation (e.g., *Heterodera trifolii*, followed by *Meloidogyne* sp. and then *Pratylenchus* spp. in white clover roots) are mechanisms by which tylenchid plant-feeding nematodes coexist in one plant but are separated in space or time.

Among three coexisting species of predacious Mononchida it has been suggested body and stoma dimensions, and thus the soil pores accessed and the size of potential food items, reduce competition between the 15 juvenile and adult stages (Yeates 1987a).

Populations of microbial-feeding nematodes may show different population growth rates on different food species (e.g., Pillai and Taylor 1967; Schiemer et al. 1980; Schiemer 1982) and have different effects on the growth of those microbes *in vitro* (e.g., Arnold and Blake 1968; Giannakis and Sanders 1989). New experimental designs have allowed demonstration of distinct food preferences by the fungal-feeding forms such as *Aphelenchus*, *Aphelenchoides* (Aphelenchida), and *Filenchus* (Tylenchida) (Ruess et al. 2000; Okada and Kadota 2003). Among bacterial-feeding Rhabditida, Venette and Ferris (1998) have shown remarkably different population growth rates on different bacterial isolates (table 12.4). In bacterial-feeding *Caenorhabditis elegans* (Rhabditida) and *Plectus palustris* (Araeolaimida), egg production has been shown to vary with bacterial cell concentrations (table 12.5). Some bacterial-feeding nematodes show remarkable elaboration of the head region (e.g., fig. 12.1); the functional significance of these modifications is gradually being understood (Moens et al. 2004).

Only after many years of study did differential breeding on host plants reveal the distinction between *Globodera rostochiensis* and *G. pallida* (Stone 1973). Among bacterial-feeding nematodes similar sibling species, such as those found in *Panagrolaimus* by Sohlenius (1988), are now being

TABLE 12.4
Influence of Bacterial Isolates on the Population Growth Rate (λ day⁻¹) of Six Nematode Species

	<i>Streptomyces halstedii Scabies</i>	<i>Brevibacterium linens</i>	<i>Escherichia Coli</i>	<i>Bacillus megaterium #B</i>	<i>Bacillus polymyxa</i>	<i>Bacillus megaterium #A</i>
<i>Rhabditis cucumeris</i>	1.0	2.7	3.8	4.0	4.5	4.7
<i>Caenorhabiotis elegans</i>	1.0	4.0	6.8	3.8	12.0	4.2
<i>Bursilla labiata</i>	1.0	1.6	2.0	2.0	1.7	1.7
<i>Cephalobus persignis</i>	1.0	1.5	1.4	1.3	1.5	1.7
<i>Acrobeloides buetschlii</i>	1.0	1.4	1.4	1.4	1.6	1.7
<i>A. bodenheimeri</i>	1.0	1.2	1.4	1.3	1.4	1.3

Note: The bacteria are in order increasing multiplication of *Rhabditis cucumeris* ($\lambda = 1$ corresponds to no increase) to illustrate differing optima on bacterial lawns at 20°C. The upper three species are Rhabditidae and show greater population growth rates than do the lower three species of Cephalobidae.

After Venette and Ferris (1998)

TABLE 12.5
Effect of Increasing Bacterial Cell Concentration on Egg Production by Two Bacterial-Feeding Nematodes

Nematode	Bacterial cells ml ⁻¹	Total eggs per ♀	Nematode	Bacterial cells ml ⁻¹	Eggs per ♀ per day
<i>Caenorhabditis elegans</i>	2×10^8	13.1	<i>Plectus palustris</i>	8×10^7	0
<i>Caenorhabditis elegans</i>	5×10^8	50.1	<i>Plectus palustris</i>	8×10^8	12.6
<i>Caenorhabditis elegans</i>	10^9	83.1	<i>Plectus palustris</i>	8×10^9	37.7
<i>Caenorhabditis elegans</i>	5×10^9	134			
<i>Caenorhabditis elegans</i>	10^{10}	145			
<i>Caenorhabditis elegans</i>	5×10^{10}	153			

After Schiemer et al. (1980), Schiemer (1982)

recognized. As molecular techniques are more widely applied many more morphologically indistinct, cryptic species are likely to be distinguished on biological bases.

12.4 SPECIES DIVERSITY AND FUNCTIONAL DIVERSITY OF NEMATODE ASSEMBLAGES

Over 400 nematode species have been recorded from individual natural or semi-natural terrestrial habitats (table 12.6). In agricultural production systems most quantitative estimates of nematode abundance identify specimens only at the generic level and table 12.7 summarizes information from 71 sites. While up to 95 species were recorded from a single site, on average the assemblages studied contained 28 nematode genera. On the basis of the proportional contribution of these genera, the Shannon-Weiner index of diversity (H') ranged from 1.1 to 4.0 (mean 2.5). Ignoring any seasonal differences, the average abundance of nematodes was 3 million m⁻². In pathogenic situations a single species may dominate the nematode assemblage; Thorne (1927) recorded 93% of the 2.8–3.7

TABLE 12.6
Records of High Nematode Species Diversity Under Natural and Semi-Natural Vegetation

Vegetation	Total nematode species	Locality	Reference
Primary tropical forest	91/431 ^a	Mbal Mayo, Cameroon	Bloemers et al. 1997
Native prairie	228	Kansas, USA	Orr and Dickerson 1966
Forest soils	125/182 ^a	Slovakia	Šály 1985
Forest woodlots	175	Indiana, USA	Johnson et al. 1972
Chalk grassland	154	Southern England	Hodda and Wanless 1994
Rainforest	153	Korup, Cameroon	Price and Siddiqi 1994

^a regional diversity, including a range of sites/management regimes

TABLE 12.7
Various Measures of Diversity in 71 Nematode Assemblages from Agroecosystems

Parameter	Mean	Range	Feeding groups ≥ 0.2	Sites at which feeding group greatest proportion
Genera listed (n = 67)	28	10–49	—	—
Species listed (n = 4)	88	39–95	—	—
Nematode abundance (thousands m ⁻²)	3,083	230–21,000	—	—
H'	2.511	1.100–3.995	—	—
Proportions by feeding groups				
plant-feeding	0.211	0–0.588	31	14
plant-associated	0.130	0–0.375	12	2
fungal-feeding	0.105	0.007–0.410	8	1
bacterial-feeding	0.397	0.126–0.789	68	47
predacious	0.035	0–0.299	1	0
“dorylaimid omnivores”	0.121	0–0.487	14	7
NCR	0.793	0.400–1.00	—	—
NGR	9.15	0–93.4	—	—

million nematodes m⁻² in two Utah sugar-beet fields as *Heterodera schachtii*. Clearly pathogenic situations, the application of organic matter, fertilizers, and other agrochemicals all affect the size and relative diversity of nematode assemblages. While some comparative series are included, most of the 71 sites summarized in table 12.7 are typical agricultural production systems.

The presence and abundance of each species in the nematode assemblage of a given agricultural production system is dependent on

1. the natural or human-mediated occurrence of the species in the region;
2. the presence of a suitable food resource;
3. environmental conditions such as temperature, moisture, soil texture/structure;
4. management factors such as plant species and variety, fertilizer, cultivation; and
5. biological interactions including succession, predation, and competition.

A variety of indices has been proposed to summarize the composition of nematode assemblages. Following Yeates (2003) the following are used:

Shannon-Weiner index of diversity

$$H' = - \sum_{i=1}^s p_i \log_e p_i$$

Nematode Channel Ratio

$$\text{NCR} = B / (B + F)$$

where *p* is the proportion of individuals in the *i*th taxon, and B and F are the relative contributions of bacterial-feeding and fungal-feeding nematodes to total nematode abundance. Thus NCR indicates the relative contributions of the bacterial- and fungal-mediated decomposition channels. To complement NCR, the nematode grazing ration [NGR = (B+F) / (P+O)] is used to indicate the ration of microbial-grazing nematodes (B+F) to predacious and omnivorous nematodes (P+O) grazing on microbial-grazers.

The loss of crop yield arising from the interaction of plant-feeding nematodes and other stresses on plants is widely known, and typical relationships are shown in figure 12.2. In contrast, the total abundance of plant and soil nematodes has also been positively correlated with plant growth in various studies (fig. 12.3). However, correlations with the populations of component nematode feeding types vary. The various plant-feeding nematodes in 15 grazed New Zealand pastures were positively correlated with herbage growth (table 12.8). In contrast, in 13 Polish grasslands large populations of *Pratylenchus* that dominated the plant-feeding nematodes were apparently pathogenic, showing a negative correlation with root growth (table 12.8). *Pratylenchus*, however, may, like other plant-feeding nematodes, be plant-pathogenic in some New Zealand pasture situations (Yeates 1976). Total nematode abundance was correlated with site productivity only in New Zealand. Nematode diversity was not correlated with productivity in either country (table 12.8); both Yeates (1984) and Ruess (2003) have stressed the dominance of soil (and climate) over management practices in determining the composition of the nematode assemblage. What is common to these New Zealand and Polish series is a significant correlation between the proportion of bacterial-feeding nematodes and site productivity (table 12.8); this is in accordance with the results of Ingham et al. (1985) discussed above. Furthermore, there was a significant correlation between NCR (i.e., the relative contribution of bacterial-feeding nematodes to decomposition processes) and site productivity (table 12.8). This suggests the dominance of bacterial-mediated decomposition processes over the slower fungal-mediated processes (Wardle 2002).

In the survey of 71 nematode assemblages, an average of 39.7% of nematodes was regarded as bacterial-feeding (table 12.7). This was greater than the 21% regarded as plant-feeding (i.e.,

TABLE 12.8
Correlations Between “Plant Growth” and Components of the Nematode Assemblages Under Grassland in Two Studies

	Poland Mown grasslands (<i>n</i> = 15) Root growth (over 7 months)	New Zealand Grazed pastures (<i>n</i> = 13) Herbage production (annual)
H' diversity	−0.176	−0.310
NCR	+0.532*	+0.525*
Nematode abundance		
Bacterial-feeding	+0.575*	+0.757*
Fungal-feeding	−0.332	+0.288
Predacious	+0.370	+0.645*
Plant-feeding	−0.664*	+0.777*
Plant-associated	+0.372	+0.662*
“Dorylaimid omnivores”	+0.410	+0.545*
Total	−0.379	+0.783*

* correlations significant at *p* = 0.05

Derived from Wasilewska (1999), Yeates (1984).

potentially plant-pathogenic) and 11% regarded as fungal feeders. Furthermore, bacterial-feeding nematodes were the only feeding group to have a significant “minimum” population (13%), and at 68 of the 71 sites they exceeded 20% of total nematodes. Indeed, at 47 of the sites they were the dominant feeding group (table 12.7). While predacious nematodes averaged only 3.5%, when aggregated with “dorylaimid omnivores” the average contribution was 16%. In these agroecosystems NCR ranged from 0.40 to 1 (i.e., no fungal feeders) and averaged 0.79. Bacterial-feeding nematodes clearly dominate most systems and decomposition, if the relative abundance of bacterial and fungal-feeding nematodes indicates their contribution to soil processes. Values in an earlier survey of 116 sites also showed that bacterial-feeding nematodes make a major contribution to nematode assemblages (43%), NCR averaging 0.77 (Yeates 2003).

A recent study extrapolated survey data from 70 Dutch dairy farms and found nematode functional diversity to decrease with increasing management intensity (Mulder et al. 2003). They regarded many taxa as endangered “as even cosmopolitan, unspecialised nematodes such as the Teratocephalidae appear suppressed under intensive management”; *Metateratocephalus* and *Teratocephalus* showed a positive influence of organic manuring. Diversity of bacterial- and fungal-feeding nematodes were found to be strictly related to cattle pressure while soil microbial biomass was greatest under organic farming. The Dutch results were comparable with those of Porazinska et al. (1999) from central Florida, who considered the unique contributions of particular nematode genera to soil ecosystem processes implied a need for generic- or species-level resolution. However, when the composition of the native nematode assemblage, local soil texture, mineralogy and chemistry, and climate are coupled with findings on the predominant, controlling effects of certain feeding/functional groups (e.g., De Deyn et al. 2003; Wardle et al. 2003a, 2003b), it seems that diversity *within* these functional groups may be the key to understanding the global impacts of agricultural production systems on nematode diversity. In the traditional, plant-pathogenic nematode view, this can be likened to the diversity of plant-feeding nematodes associated with a field of a particular crop and able to induce economic crop loss when additional stresses affect the plants.

Replicated sampling is used to obtain “average” nematode populations on a paddock scale. However, most resources and processes occur in discrete “patches,” ranging from root tips to buried pockets of dead plant material (e.g., 3,000 nematodes g⁻¹ dry matter; Sohlenius and Boström 1984). Bulk and rhizosphere soil differ in their nematode assemblages (Hofman and s’Jacob 1989), and across a paddock the assemblages vary with plant species, crop rows, and soil factors (Wyse-Pester et al. 2002; Ettema and Yeates 2003). The distribution of particular nematodes underlie these patterns. Among the bacterial-feeders Rhabditidae depend more on patchily distributed, high bacterial densities than do Cephalobidae (Bouwman and Zwart 1994). Trichodoridae and various migratory root-feeding nematodes feed on epidermal cells, sedentary ectoparasites such as *Criconema* feed on cells within roots, and sedentary endoparasites such as Heteroderidae induce transfer cells adjacent to the vascular bundle (Hussey and Grundler 1998).

In agroecosystems there are typically many species in each nematode feeding or functional group, and their relative abundance varies during the cropping cycle. In an effort to better understand ecosystem processes, several recent field studies have demonstrated statistically significant effects of functional groups (e.g., De Deyn et al. 2003; Mulder et al. 2003; Wardle et al. 2003a, 2003b). Using a simulation model, Hunt and Wall (2002) showed that extinction of root-feeding nematodes was one of few perturbations of soil biodiversity that impacted on overall ecosystem function by at least 10%. This, however, was a simulated removal of a functional group and made no allowance for complementarity that might occur within a nematode functional group if a species-level taxon was lost.

12.5 MANAGEMENT PRACTICES AND THE NEMATODE ASSEMBLAGE

Most agricultural management practices are intended to affect final crop yield, and, presumably, their relationships to plant and soil nematodes are through the soil, plant growth, and decomposition processes that influence this yield. Clearly when root-feeding nematodes reach damaging levels

their effect on yield can be measured and they can be regarded as plant-pathogens (e.g., fig. 12.2). The following paragraphs discuss situations in which medium- to long-term management has substantially changed yield and nematode assemblages in agroecosystems.

In New Zealand, 34 years' cropping of Kairanga soil resulted in an increase in soil bulk density from 1.03 to 1.21 Mg m⁻³ with a concurrent decline in soil organic content (table 12.9), an adverse effect on crop yield, and an increased cost of soil tillage (Scrimgeour and Shepherd 1998). There was a decline in the number of nematode genera recovered, in total nematode abundance, and in H', the first 5 years having the most pronounced effect. Bacterial-feeding nematodes were the greatest contributor to the nematode assemblage in all stages, but after 34 years there was a marked increase in predacious nematodes (table 12.9). In Marton soil there was a similar trend in soil bulk density (and both yield and cultivation cost), with nematode diversity again dropping markedly. The proportion of bacterial-feeding nematodes increased over the 20 years of cropping. The increase in predacious nematodes after 34 years of cropping appears to be associated with structural changes in both soil and decomposition pathways. Yeates et al. (1998) suggested that, with decline in soil structure, predacious nematodes may graze on the microbial/protozoan lawn on buried plant residues between large soil units ("clods") rather than within smaller units ("peds"). This is consistent with the differential influence of soil structure on plant-feeding nematodes such as *Xiphinema*, *Longidorus*, *Trichodorus*, and *Heterodera* reported by Jones et al. (1969).

In a long-term study of the effects of pollutants in The Netherlands, soil liming and copper addition were used to manipulate bioactive copper (Korthals et al. 1996). Considering eight of the treatments, there was a significant decline in maize yield and nematode diversity with increasing bioactive copper (fig. 12.4A, fig. 12.4B). However, nematode diversity was positively correlated with

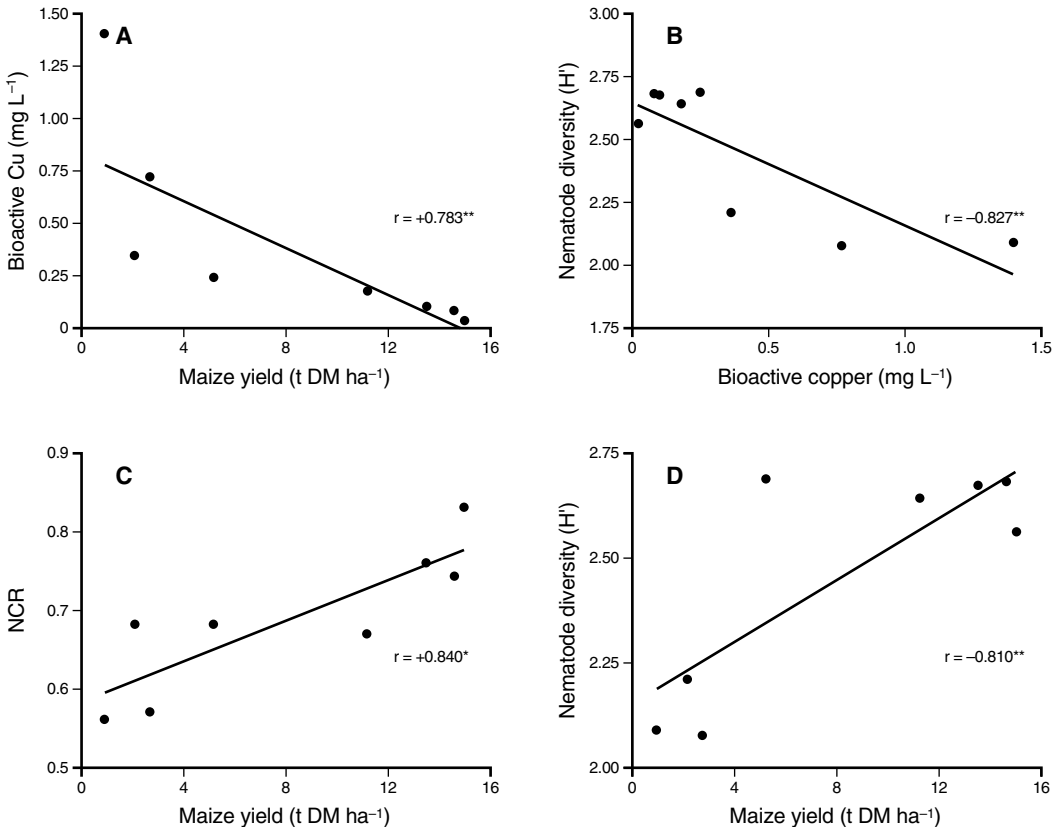


FIGURE 12.4 Results from a long-term field trial in which lime and copper sulphate were used to adjust soil pH and bioactive copper. Graphs have been derived from Korthals et al. (1996).

TABLE 12.9
Soil Conditions and Nematode Assemblages at 0–10 cm Depth in Two Cropping Soils in New Zealand (Yeates et al. 1998). In the Lower Panel Feeding Types that Represent >20% of the Nematode Assemblage Under that Soil Management are in Bold.

Soil	Management	Soil bulk density (Mg m ⁻³)	Soil organic C (g kg ⁻¹ soil)	Nematode abundance (thousands m ⁻²)	NCR	H'	Proportions in feeding types					
							Plant-feeding	Plant-associated	Fungal-feeding	Bacterial-feeding	Predacious	Dorylaimid omnivores
Kairanga	Permanent pasture	1.03	49.9	2817	0.931	2.09						0.190
	5 y cropping	1.06	35.0	1859	0.861	2.03						0.178
	16 y cropping	1.13	33.0	1861	0.946	1.43						0.099
	34 yr cropping	1.21	20.3	1596	1.000	1.95						0.169
Marton	Permanent pasture	1.05	42.8	2749	0.888	2.20						
	7 y cropping	1.14	24.8	1749	0.719	1.95						
	20 y cropping	1.21	27.2	1972	0.924	1.95						
Kairanga	Permanent pasture	0.147	0.052	0.029	0.515					0.066		
	5 y cropping	0.002	0.148	0.077	0.519					0.076		
	16 y cropping	0.012	0.134	0.041	0.687					0.027		
	34 yr cropping	0	0.128	0.050	0.355					0.299		
Marton	Permanent pasture	0.222	0.106	0.029	0.229					0.113		0.302
	7 y cropping	0.008	0.213	0.116	0.315					0.021		0.326
	20 y cropping	0.042	0.346	0.036	0.435					0.014		0.128

maize yield (fig. 12.4C) and the proportion of microbial-feeding nematodes that were bacterial-feeding (=NCR) was also positively correlated with maize yield (fig. 12.4D). Clearly, the negatively correlated bioactive copper and maize yield were associated with contrasting trends in the nematode assemblage.

A comparison of conventional and organic pastures on three soil types in Wales showed total nematode abundance, and both the abundance and proportional contribution of fungal-feeding nematodes to be greater under organic management (table 12.10). In two of the soil types nematode diversity was greater under organic management, while in the third soil both diversity values at Glanrhyd were greater than in the other two soils. These changes are in keeping with the slower, more fungal-dominated decomposition processes expected in the absence of fertilizer inputs (Wardle 2002), and with trends recorded in The Netherlands (Mulder et al. 2003).

When organic matter is incorporated into soil its composition affects the microbial community and the nematode assemblage that develop. Griffiths et al. (1994) found that while incorporation of farmyard manure had no detectable effect of nematode populations, incorporation of poultry manure led to a considerable increase. The increase favored bacterial-feeding nematodes and rhabditids in particular. They suggested that the more recalcitrant farmyard manure would bring about long-term changes in the nematode assemblage.

Addition of organic matter, in the form of municipal soil waste compost, had a greater effect on the nematode assemblage under citrus trees in Florida over three years than did irrigation or fertilizer application (Porazinska et al. 1999). Irrigation and fertilizer affected some nematodes sporadically, but mulch had a consistent and frequently significant effect. Rhabditidae, *Cephalobus*, *Aphelenchus*, and *Aphelenchoides* showed temporary responses to mulch addition, while *Plectus* and *Belonolaimus* were always more abundant in mulch-treated plots, which often had greater moisture content. *Acrobeles*, *Acrobeloides*, *Eucephalobus*, *Teratocephalus*, *Criconemoides*, *Aporcelaimellus*, and *Eudorylaimus* were always less abundant in mulch-treated plots. Generic measures

TABLE 12.10
Total Nematode Abundance and the Proportional Contribution of Nematode Feeding Groups in Three Welsh Pasture Soils Under Either Conventional or Organic Management. (NCR and H' are also given)

	Soil/management					
	Trawsgoed		Goodwick		Glanrhyd	
	Conventional	Organic	Conventional	Organic	Conventional	Organic
Total nematodes (million m ⁻²)	3.09	5.10	3.56	3.99	3.70	4.52
Proportion in feeding groups						
Plant feeding	0.535	0.282	0.105	0.378	0.316	0.401
Plant associated	0.145	0.298	0.235	0.171	0.238	0.170
Fungal feeding	0.082	0.099	0.034	0.110	0.108	0.144
Bacterial feeding	0.198	0.273	0.551	0.277	0.250	0.204
Predacious	0.005	0.002	0.032	0.009	0.018	0.016
Zooparasitic	0	0.002	0.011	0	0	0
Dorylaimid omnivores	0.035	0.045	0.033	0.055	0.070	0.065
NCR	0.710	0.735	0.942	0.719	0.670	0.596
NGR	8.15	19.2	11.06	8.66	5.00	5.50
H'	2.245	2.477	2.557	2.796	2.829	2.728
Abundance of bacterial-feeders	0.612	1.392	1.962	1.105	0.925	0.922
Abundance of fungal-feeders	0.253	0.505	0.121	0.439	0.400	0.651

Note: Where there is a significant ($p < 0.05$) management effect the higher value for each soil is in bold.

After Yeates et al. (1997)

of diversity and richness were confounded by complementary changes that Porazinska et al. (1999) considered to imply unique contributions of nematode genera to soil processes on a temporal scale. They considered that generic or species-level discrimination would provide the most adequate information about ecosystem processes. After seven years of weed management in maize and asparagus crops by cultivation, herbicide, or mulch, Yeates et al. (1999b) found that under sawdust mulch the proportional contribution of plant-feeding, predacious, and omnivorous nematodes was increased but plant-associated, fungal-feeding, and bacterial-feeding nematodes were reduced. This was attributed, in part, to the differing soil moisture regimes. Neither nematode diversity (H') nor NCR were altered.

Use of fertilizers in agroecosystems affects plant growth and through increased growth, higher nutrient status, thicker plant cell walls, increased rhizodeposition, etc., all components of the nematode assemblage are affected (Yeates 1987b). Some studies of fertilizer rates compare organic and synthetic forms of fertilizer nitrogen and include counts of plant-feeding and free-living nematodes (e.g., Akhtar 2000). In California it was found that a year after disruptive (i.e., intensive) soil management the effects on nematode feeding groups other than plant feeders had disappeared (Berkelmans et al. 2003). Over six years, Boag et al. (1998) found that removal of conventional agricultural management at three lower input system sites in England showed no evidence of either a positive or negative impact of the reduced application of agrochemicals on the nontarget plant-parasitic nematode populations; the greatest differences were the year-to-year variability in nematode numbers for which there was no obvious explanation. Like many, this study assessed change in nematodes when management-induced "stress" was removed; the question of what changes in diversity occur when such "stresses" are imposed was not addressed. In general, then, there are no data on the direct effects of agrochemicals on nematode biodiversity.

Long-term effects of weed management strategies on maize and asparagus crops were reported by Yeates et al. (1999b) and the effect of sawdust mulch has been discussed above. Yeates et al. (1999b, fig. 7) illustrate changes in nematode diversity (H') over seven years. Generally diversity under the five treatments varied in a similar pattern with time. There was a tendency for H' in 0–5 cm soil under asparagus to diverge from that under maize in the latter four years, but differences were generally not significant. Four to eight years of herbicide usage in a Slovak vineyard were associated with marked reduction (36–95%) in total, of autumn nematode populations (Šály 1989), but data on both weed growth and nematode diversity are lacking.

Some plants, including many Compositae, have nematocidal properties. When such antagonistic plants are intercropped or grown as a cover crop, they can reduce economic loss from plant-feeding nematodes. Marigolds (*Tagetes* spp.), *Crotalaria*, and asparagus (*Asparagus officinalis*) are widely quoted but sesame (*Sesamum indicum*), mustard (*Brassica* spp.), and *Sphenoclea zeylanica* have similar effects (Bridge 1987). The pathways through which the isocyanates, cyanogenic glycosides, thiopenes, and other active compounds act on nematodes are poorly known; they are generally screened against only economically important plant-feeding nematodes (Chitwood 2002) and overall nematode diversity is not assessed. A seven-year weed management study under maize and asparagus showed that for comparable treatments and soil depths nematode diversity was consistently lower under asparagus than under maize (table 12.11). Although there are some published results for the abundance of plant-feeding, predacious, and "free-living" nematodes, the effects of antagonistic plants on various nematode feeding groups are largely unstudied.

As plant and soil nematodes live and move in soil water films, pore size, soil texture and soil structure significantly affect their movement, access to food resources, and biological activity (Blackshaw and Senthamizhselvan 1991; Bouwman and Arts 2000; Yeates et al. 2002; Elliott et al. 1985; Jones et al. 1969). The soil in a particular agroecosystem has a significant effect in determining the nematode assemblage present, and while many management practices have relatively little impact on the composition of the assemblage (Yeates 1984), planting crops susceptible to certain nematodes (e.g., sugar beet and *Heterodera schachtii*), intensive cultivation that affects soil structure (table 12.9), addition of toxins (fig. 12.4), etc., can all impact on local nematode diversity. A sandy

TABLE 12.11
Nematode Diversity (H') in 0–5 and 5–10 cm Soil Under Maize and Asparagus Crops After Five Weed Management Treatments Were Applied for Seven Years

Treatment	Maize		Asparagus	
	0–5 cm	5–10 cm	0–5 cm	5–10 cm
Sawdust	1.86	1.85	1.68	1.07
Cultivated	1.89	1.92	1.66	1.68
Hand-hoed	2.17	1.96	1.85	1.43
Atrazine/Caragard	2.00	1.93	1.48	1.42
Sulfonylurea/Bromacil	1.95	1.77	1.41	1.44
Mean	1.97	1.89	1.62	1.41

After Yeates et al. (1999b)

TABLE 12.12
Nematode Diversity (H') Under Pasture and Two Intensities of Wheat Cultivation in South Australia

	Sandy soil (Avon)	Silty soil (Kapunda)
Pasture	2.36	1.987
Direct drilled wheat	1.85	1.86
Conventionally cultivated wheat	1.94	1.51

After Yeates and Bird (1994)

TABLE 12.13
Nematode Diversity In and Between Rows of Soybean (*Glycines max*) at Different Times After Sowing

	Row	Inter-row
5 June (planting)	2.28	2.37
14 July	2.52	2.31
20 August	2.13	2.52*
1 October	2.25	2.45

* indicates a $p < 0.05$ difference between positions on that date

After McSorley and Frederick (1996)

soil in South Australia generally had a more diverse nematode assemblage under wheat than a nearby silty soil (table 12.12), but the diversity was less than that under pasture. In Florida, nematode diversity in a soybean field changed during the growing season and, on one sampling date, was significantly greater between crop rows than within crop rows (table 12.13).

Studies from a range of crops, soils, and climate show that nematode assemblages in agroecosystems are abundant, diverse, and dynamic. Nematode distribution is patchy, within both paddocks

and regions. Many agricultural practices affect nematodes and after several years of constant practices there may be a shift in the average nematode assemblage in a paddock. However, many changes are transitory, and unless chemical (e.g., heavy metals) or soil structural conditions alter drastically, the assemblages are sufficiently resilient to recover.

12.6 OVERVIEW

1. Soil nematode activity not only causes reduction in crop yield, but nematode grazing on microbial populations can increase fluxes of plant nutrients in soils. Just as plant-feeding nematodes differ in their effects on plants and plant growth, microbial-feeding nematodes differ in the microbes they utilize and the fluxes they affect.
2. Soil nematode assemblages are abundant and diverse. The specificity of nematode feeding and effects on soil processes lead to correlations between nematode diversity and crop yield but, as in many ecological situations, direct causation is difficult to demonstrate.
3. Soil nematode assemblages appear resilient to many agroecosystem management practices. However, most analyses are at the generic level and there may be greater changes, or taxon replacement, at the species level.
4. While many management regimes appear benign, long-term tillage can lead to change in soil structure and in the makeup of soil nematode assemblages. There is evidence that antagonistic plants (e.g., asparagus) may affect diversity of the nematode assemblage rather than simply reducing plant-feeding nematodes.
5. Under organic management the proportion of fungal-feeding nematodes has been found to increase, in keeping with ideas on slower, fungal-mediated decomposition processes. While incorporation of organic matter often changes soil nematode assemblages, complementary changes among species mean that overall diversity may be unchanged. Changes when stubble or compost is added may be driven by tillage-induced changes in soil structure and water characteristics as much as by the organic inputs themselves.

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13 Diversity of Tardigrada

Kunihiro Seki and Daiki D. Horikawa

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13.1 INTRODUCTION

Tardigrades, often referred to as “water bears,” are classified in the phylum Tardigrada. They have lobopodous legs, and most are 250–500 μm in length as adults.^{1,2} The habitat of these small animals ranges from sea to fresh water to land, and they have been collected from ocean depths of 4,960 m to altitudes of 6,600 m in the Himalayas.^{1,2} The number of species is said to exceed 800.³

All tardigrades, however, must be active in water, even terrestrial species. For this reason tardigrades are fundamentally defined as aquatic animals. Terrestrial tardigrades inhabit microenvironments such as in lichens, mosses, soil, and leaf litter, and in such environments they are often exposed to periods of dehydration. In these environments, tardigrades often suspend any activities. This state of suspended activity is called cryptobiosis, and it is a characteristic strategy of terrestrial tardigrades.

This chapter mainly examines the survival strategy of terrestrial tardigrades.

13.2 CRYPTOBIOSIS

Cryptobiosis has been defined as latent life, in which an organism ceases showing biological responses and metabolism is reduced to an undetectable level.⁴ Cryptobiosis may be classified into four categories depending on the environmental stress factor. These are anhydrosis induced by dehydration, cryobiosis induced by low temperature, anoxybiosis induced by lack of oxygen, and

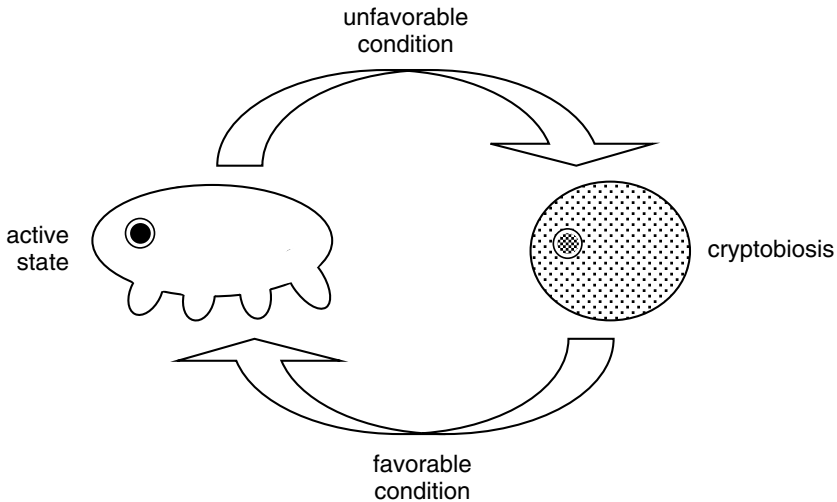


FIGURE 13.1 A cycle of active and inactive state (cryptobiosis) in a tardigrade.

osmobiosis induced by high salt concentration.⁴ Cryptobiosis is brought about by changes in the habitat of individuals that make the environment inhospitable. When the environment returns to a favorable condition, the individuals are released from the cryptobiotic state and resume lifecycle activities of growth, foraging, and reproduction (fig. 13.1).

Among Tardigrada, the most primitive are considered to be the marine order Arthrotardigrada, which do not have any types of cryptobiosis described above.⁵ This is thought to be because marine environments are comparatively stable, making adaptation to sudden environmental changes unnecessary. Whether tardigrades have all or some of these cryptobiotic abilities differ with the species.

Among the four types of cryptobiosis, anhydrobiosis has been the focus of most attention, and has been studied over 300 years since Leeuwenhoek. Since Tardigrades are essentially aquatic animals, it is difficult to imagine that they would have made such progress on land without acquiring the capacity for anhydrobiosis.

A great diversity of tardigrade species are also seen in the polar regions of the Arctic and Antarctic, but adaptation to these cold environments must depend in large part on cryobiosis.

With this as background, this chapter examines the two cryptobiosis categories of anhydrobiosis and cryobiosis, so important for terrestrial tardigrades.

13.2.1 ANHYDROBIOSIS

A phylogenetically diverse range of organisms—plant seeds, bacterial and fungal spores, nematodes, rotifers, tardigrades, eggs of brine shrimp, and one species of chironomid in Diptera—possess the capacity for anhydrobiosis.⁶

Terrestrial tardigrades go about the activities of feeding and reproduction when there is water from rain or other sources. When their surroundings become dry they fall into a state of inactivity in which all movement ceases. This state continues until the next opportunity to obtain water. The inclusion of this stage of anhydrobiosis in their life history is a major characteristic of terrestrial tardigrades.

Research on anhydrobiosis in tardigrades has focused mainly on the physiological and biochemical aspects, although there has not been as much research even in these areas on tardigrades as on nematodes. Research on anhydrobiosis in tardigrades since the start of the 20th century has included the studies of Rahm in the 1920s, Pigon and Weglarska in the 1950s, Crowe and colleagues in the 1960s through 1970s, and Wright in the 1980s. Since the 1990s there have been the studies of Westh and Ramløv and Jönsson and colleagues. All have contributed significant findings.

13.2.1.1 Induction of Anhydrobiosis

In natural environments, water evaporates very slowly within microenvironments such as moss, lichens, or soil even when the surrounding areas have become strongly dehydrated. These microenvironments maintain a high relative humidity and dehydrate slowly. For this reason, the area surrounding an individual tardigrade also maintains high humidity in dehydration; in fact, to induce complete anhydrobiosis in tardigrades requires dehydration with high relative humidity. As tardigrades go further into anhydrosis their bodies shorten on the longitudinal axis with folding of the exterior cuticle and invagination of the legs. This is called a “tun” state, when tardigrades change form into a barrel shape^{1,5} (fig. 13.2).

In *Macrobiotus areolatus*, nearly all individuals form a tun state when subjected to a dehydration process with relative humidity of 70% or more and are revived with the addition of water. When dehydrated under a relative humidity of 0%, they do not go into a tun state but simply die.⁷ When tardigrades are dehydrated under a high relative humidity, the loss of water from the body is slow. Conversely, when dehydrated under conditions of low humidity the body rapidly loses water.⁷ Thus, tardigrades seem to require an adequate physiological preparation time in order to enter anhydrobiosis.

In the chironomid *Polypedilum vanderplanki*, anhydrobiosis is not induced through the brain or nervous system.⁸ Anhydrobiosis in tardigrades also may therefore be a response to dehydration stress at the tissue or cell level.

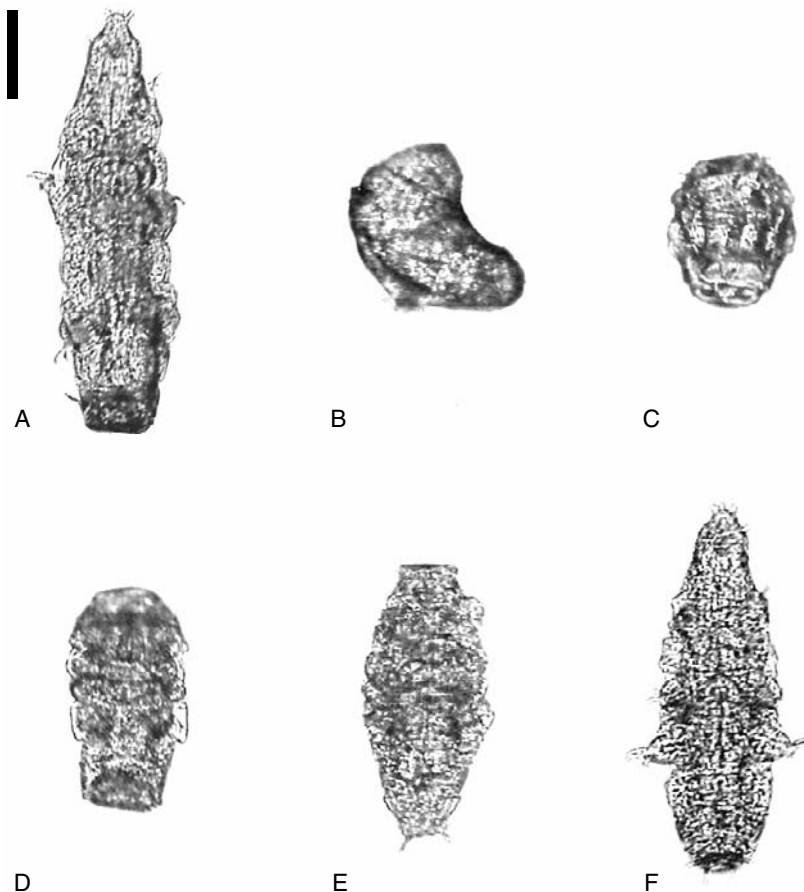


FIGURE 13.2 Transition to anhydrobiosis (A–C) and recovery from this state (C–F) in *Milnesium tardigradum*. An anhydrobiotic tardigrade shows tun formation (C). Scale bar, 100µm.

13.2.1.2 Metabolic State and Longevity of Anhydrobiosis

When *Milnesium tardigradum* was reared in a laboratory environment so that it did not enter anhydrobiosis, the maximum longevity was 57 days.⁹ Another report shows that tardigrades in an anhydrobiotic state in dehydrated moss kept for 120 years in a museum became active again after receiving water.¹⁰ Thus, debate continues as to whether metabolism has completely stopped in individuals in an anhydrobiotic state. In a study by Pigon and Weglarska,¹¹ metabolic activity in the tardigrade *Macrobiotus hufelandi* in anhydrobiosis was shown to drop to a minimum of 0.035% in an active state, rather than to cease altogether. However, it seems that the amount of respiration increases with the surrounding relative humidity.

Anhydrobiotic eggs of *Ramazzottius oberhauseri* were successfully hatched after being stored at ambient temperatures for 9 years, but no adults or juveniles were revived after being kept for the same period.¹² This suggests that eggs can continue living for a longer time in a state of anhydrobiosis. Even so, dehydrated individuals of *Echiniscus jenningsi*, *Macrobiotus furciger*, and *Diphascon chilense* showed survival rates of 14.9%, 51.3%, and 41.8%, respectively, when kept for about 8 years at -22°C .¹³

From the above one may conjecture that metabolism is elevated and aging promoted in anhydrobiotic tardigrades kept under high humidity conditions, and the organism may be damaged by free radical oxidation if kept under conditions of the presence of oxygen. It may therefore be that viability is maintained for a longer period under low humidity and anoxic conditions. It is also seen that viability continues for a longer period when tardigrades are kept under low temperature.

13.2.1.3 Sugar and Protein Accumulation

In most cases when an organism is exposed to dehydration stress and its cells lose water, the cells suffer irreversible mechanical damage, ultimately resulting in death of the organism. Most anhydrobiotic organisms are known to synthesize the nonreducing disaccharide trehalose.¹⁴ The tardigrade *Adorybiotus coronifer* begins accumulating trehalose within the body as anhydrobiosis is induced, with the final concentration reaching 23 times that in the active state.¹⁵ Trehalose is thought to act in place of water to maintain the structure of biological membranes and proteins,¹⁶ thereby preserving the biological structure of organisms that have entered anhydrobiosis and promising their revitalization. This is called the “water replacement hypothesis,” and it is supported by numerous researchers. Conversely, another frequently mentioned hypothesis refers to trehalose’s role of “vitrification,” in which cell components are preserved by being suspended in high-viscosity glass.¹⁷

The amount of trehalose produced varies from organism to organism, accounting for 12–13% of the dry weight of the nematode *Aphelencus avenae*¹⁸ and 18% of the chironomid *Polypedilum vanderplanki*,⁸ in contrast, the amount of trehalose synthesized in the tardigrade *A. coronifer* is only 2.3%.¹⁵ Moreover, the rotifers *Philodina roseola* and *Adineta vega* lack the gene associated with trehalose synthesis, and anhydrobiosis is known to be induced in these organisms without the synthesis of any trehalose whatsoever.¹⁹ Thus, synthesis of trehalose is not essential for animals to enter anhydrobiosis, suggesting the possibility that even in those animals that do synthesize trehalose, the realization of anhydrobiosis does not depend on trehalose alone.

It is known that the tardigrade *M. tardigradum* synthesizes hsp70, one type of heat shock protein, when it enters anhydrobiosis.²⁰ It is also known that the hsp70 protein, as the chaperonin, plays roles in the folding of newly synthesized polypeptides within the cell and in preventing denaturation of proteins owing to heat stress.²¹ One may reasonably conclude that this type of protein contributes in some way to anhydrobiosis in tardigrades, but it will probably be some time before the role is fully clarified. In the nematode *A. avenae*, a gene has been found that codes for late embryogenesis abundant (LEA) protein, which until that time had been observed only in plants.²² This indicates that the same mechanism acts in the induction of anhydrobiosis in both

plants and animals. This LEA protein coils and becomes a higher order filamentous structure due to dehydration, and accumulates within dehydrated cells. It has been suggested that LEA protein acts to maintain the mechanical structure of dehydrated cells²³ or to stabilize trehalose glasses and protect biological molecules,²⁰ but its detailed actions remain unclear. It would seem possible that tardigrades also synthesize LEA protein and that it is involved in the completion of anhydrobiosis. Further research may clarify this issue in the near future.

From the above it seems that in anhydrobiosis in tardigrades constructional systems of mainly trehalose and proteins are expressed, which act synergistically to protect the organism components from damage due to dehydration.

13.2.1.4 Revival from Anhydrobiosis

Generally, if organisms showing anhydrobiosis rehydrate under low relative humidity environments, cell solutes leak and the organism suffers mechanical damage. As a result the organism cannot be readily revived, leading to death.¹⁴ For example, among nematodes,^{24,25} plant seeds,⁶ and bacteria,²⁶ complete revival in many organisms can be guaranteed with prehydration at high relative humidity before rehydration with water.

However, among terrestrial tardigrades are species with strong desiccation tolerance, and it has been observed by some researchers that these species can recover from anhydrobiosis even without the need for prehydration.²⁷ In a recent preliminary experiment, *M. tardigradum* were observed to revive with no damage even when rehydrated under a relative humidity of 0% (Horikawa & Higashi, unpublished data). Highly desiccation tolerant tardigrades may have developed resistance to both rapid dehydration and rapid rehydration in order to adapt to very dry habitats. However, there has been very little research in this area, and further developments are eagerly awaited.

13.2.1.5 Ecological Significance of Anhydrobiosis

There is interspecific variation in the degree of desiccation tolerance of tardigrades, and some species can enter anhydrobiosis under conditions of extreme desiccation—for example, *R. oberhauseri* and *M. tardigradum*—while in others, such as *Hypsibius prosostomus* and *H. dujardini*, anhydrobiosis is not induced well unless humidity is high.²⁸ Moreover, the same report shows that water loss from the organism during dehydration is suppressed in species with stronger desiccation resistance, and that there is a positive correlation between strength of desiccation resistance and degree of surface area reduction during tun formation between species of Eutardigrada.²⁸

While it is possible that there is some variation between within-species populations in the trait of desiccation tolerance, a comparison of desiccation tolerance between populations of two species of tardigrade (*A. coronifer* and *R. oberhauseri*) in Italy and Sweden revealed no such variation.²⁹ However, a difference was found in anhydrobiotic capacity between populations of *M. tardigradum* in Japan and Indonesia (Horikawa & Higashi, unpublished data). It has also been suggested that the body size (i.e., the age and developmental stage) of the organism is related to the strength of its desiccation tolerance.^{12,29,30}

Also suggested is that the strength of the desiccation tolerance of terrestrial tardigrades reflects the environment in which a species lives.^{28,31} Thus, species inhabiting very dry environments must have stronger desiccation tolerance than those inhabiting humid environments, and enter anhydrobiosis even under relatively low humidity. Desiccation tolerance is suggested to differ in strength depending on the water conditions in a habitat, and attempts have been made to classify tardigrades according to their preference for these water conditions.^{1,31}

If desiccation stress is taken to be a definitive selective pressure promoting speciation among tardigrades, it would also be easier to explain the habitat preference among these different species. One may be tempted to try to demonstrate this through artificial selection, but the difficulty at present of raising tardigrades in the laboratory makes this impracticable. However, there are species that

have adapted to a wide range of water conditions, and by surveying the various traits (morphology, etc.) within these species between populations living in very dry places and those living in humid environments, it may be possible to see some kind of effect of desiccation stress on these traits.

Acquiring the capacity for anhydrobiosis made it possible for terrestrial tardigrades to survive environments with no water, but this capacity of anhydrobiosis has also been suggested to have some costs. It has been observed that before entering anhydrobiosis and after reviving from anhydrobiosis the size of lipid storage cells in tardigrades become smaller.^{27,30} This is thought to be owing to the synthesis of glycerol and other substances from the lipids in these storage cells during the transition to anhydrobiosis, but this may also lead to reduced survival and reproductive capacities in individuals immediately after reviving from anhydrobiosis. Thus, there appears to be a trade-off relationship between anhydrobiosis and reproduction. Considered from this viewpoint, while terrestrial tardigrades have the trait of anhydrobiosis in all stages of the life cycle, in the egg and juvenile period there may be a bias in the energy distribution to investment in survival, whereas in adults with the ability to reproduce there is a greater investment in reproduction. Thus, the capacity for anhydrobiotic survival is expected to be higher in stages of eggs and juveniles, and then to decrease with age. Much research has been conducted in this area in recent years,^{30,32} and further results are awaited.

Terrestrial tardigrades are known to come together in one place in times of dehydration. By aggregating together the surface area of each individual that is exposed to the outer environment is reduced, which has the effect of inhibiting evaporation.²⁷ However, it is not known whether this behavior is actively carried out. It has also been reported that a certain tardigrade moved toward the bottom area in moss where more water is retained as the moss dried out,²⁹ but in a later attempt by Nelson and Adkins³³ to demonstrate this behavior in five species of tardigrade, it could not be confirmed.

13.2.1.6 Tolerance to Extreme Environments

It has been shown that tardigrades in an anhydrobiotic state can survive extreme environments, including vacuums, temperatures from -273°C to $+151^{\circ}\text{C}$, and exposure to X-rays in amounts 1000-fold that which would kill a human.^{34,35} They also have tolerance to other extreme environments, surviving exposure to high hydrostatic pressure of 600 MPa³⁶ (fig. 13.3) and fumigation with methyl bromide.³⁷ One reason for this is thought to be that individuals that have lost water to the extent that there is no free water whatsoever in the body become tolerant to various environmental loads. In

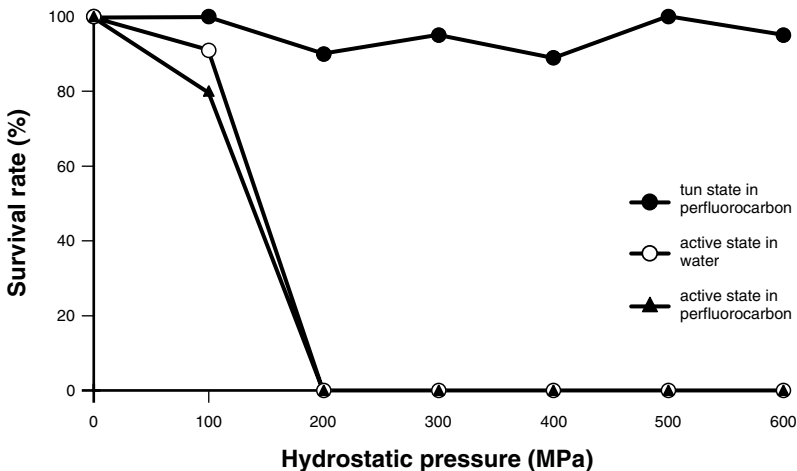


FIGURE 13.3 Survival rate of *Macrobiotus occidentalis* after exposure to hydrostatic pressure ranging from 0 to 600 MPa (redrawn from Seki & Toyoshima, 1998).

fact, when *Macrobiotus occidentalis* in anhydrobiosis was exposed to high hydrostatic pressure some dead individuals were observed and it was confirmed that water within the body had leaked.³⁶

It may also be that sugars and proteins produced specifically in anhydrobiosis protect the integrity of biological structure from the load of extreme environments, but at this stage much remains unknown.

13.2.2 CRYOBIOSIS

Cryobiosis is defined as a type of cryptobiosis that is induced by low temperature.⁴ Tardigrades that inhabit cryoconite holes formed on the surface of glaciers (listed by Sømme³⁸ are expected to regularly freeze from exposure to temperatures below the freezing point, but these species survive such an inhospitable environment through cryobiosis. *A. coronifer* in an active state entered cryobiosis with gradual cooling, and survived a low temperature of -196°C .³⁹ Moreover, Rahm discovered in 1922 that the tardigrade *R. oberhaeuseri* could survive even when the temperature was decreased to -253°C . Some researchers maintain the misconception that cryobiosis is the cold tolerance of individuals in an anhydrobiotic state, but to be precise cryobiosis is a phenomenon induced by exposure of active hydrated individuals to low temperature. Moreover, since no lethal low temperature (LLT) is seen in cryobiosis, it may be distinguished from other types of cold tolerance.^{39,40} However, there have been very few studies concerning cryobiosis compared with anhydrobiosis in tardigrades, and knowledge is limited.

13.2.2.1 Effects of Cooling Rate on Cryobiotic Survival

When *A. coronifer* was cooled to -196°C a tendency was seen for the survival rate to decrease as the cooling rate was increased. When cooled from room temperature at a rate of $1500^{\circ}\text{C}/\text{min}$, all individuals died, but survival was possible when the cooling was done for the first 30 sec or so at a rate of $30^{\circ}\text{C}/\text{min}$ followed by $1500^{\circ}\text{C}/\text{min}$.³⁹ From this it is seen that cryobiosis is induced by a relatively slow initial cooling rate. When the tardigrade *A. coronifer* was cooled the free water within individuals froze at about -6°C to -7°C , and it was found that about 80% of the water within the body froze.^{41,42}

Thus, *A. coronifer* is classified as a freeze-tolerant animal.

13.2.2.2 Effects of Cooling Periods on Survival

Three tardigrade species that inhabit the Antarctic, *E. jenningsi*, *M. furciger*, and *D. chilense*, were kept for various periods in a cryobiotic state at -80°C . No change was found in the survival rates of *M. furciger* and *D. chilense* even after a period of 150 days, whereas the survival rate of *E. jenningsi* declined rapidly if kept in this state for longer than 10 days.¹³ These results show that cold tolerance also differs among species. These species of tardigrade in an anhydrobiotic state were cryopreserved under the same conditions, and the survival rate was found to be higher. Thus, since not only the freezable water but also the nonfreezable water is eliminated from the body in anhydrobiosis, the cold tolerance is found to be greater than in the state of cryobiosis, when the body still holds some water.^{38,39}

13.2.2.3 Cryoprotectants

While there have been no direct studies of cryoprotectants in tardigrade cryobiosis, cold tolerance was found to be slightly higher in *A. coronifer* individuals in which trehalose was accumulated in the body before the induction of cryobiosis than in individuals in which it was not.³⁹ Moreover, in a similar type of cold tolerance—thought perhaps to be cryobiosis—exhibited by the nematode *Panagrolaimus davidi*, trehalose is known to accumulate in the body if cold acclimation is allowed.⁴³

The above suggests that trehalose may also be a cryoprotectant in cryobiosis, although clear evidence of this has not yet been obtained.

13.2.3 SIMILARITY BETWEEN ANHYDROBIOSIS AND CRYOBIOSIS

Both anhydrobiosis and cryobiosis are accompanied by the common phenomenon of decreasing amount of water in the body that can be used by the individual. It may be, therefore, that there is a close relation between the desiccation tolerance and cold tolerance in tardigrades.^{39,40} For example, it has been suggested that because of the strong desiccation tolerance of *A. coronifer*, namely, its capacity to withstand the loss of water from its cells, this organism can survive undamaged even when exposed to the same water loss due to freezing.³⁹ In addition, in the nematode *P. davidi*, which has an anhydrobiotic capacity, a phenomenon has been seen in which, if the humidity is slowly lowered, or if the organism is frozen at a somewhat high subzero temperature, water is lost from within the body without freezing. This has been called "cryoprotective dehydration."⁴⁴ Thus, as has been suggested by Wright,⁴⁰ animals with a capacity for anhydrobiosis may be able to similarly withstand the loss of water that accompanies freezing. However, from the viewpoint of the biochemical function of cryoprotectants, both similarities and differences exist in the actions of biological molecules in response to desiccation and freezing stress, and debate on this matter continues.^{38,40,45}

13.3 FUTURE RESEARCH ON CRYPTOBIOSIS

Attempts to apply the phenomenon of anhydrobiosis to the preservation of cells and tissues have been made since about 1990, but most of these have been attempts at dry preservation using trehalose as the preserving medium. Recently, however, a method of anhydrobiotic engineering has been developed to produce trehalose in cells that do not have desiccation tolerance with the aim of giving them this tolerance.⁴⁶ This method has resulted in increased desiccation tolerance in desiccation-sensitive bacteria.⁴⁷ At the same time, other results have shown that no change occurs in desiccation tolerance when trehalose synthesizing genes are introduced into mouse cells that then produce trehalose.⁴⁸ Trehalose alone is therefore insufficient to realize desiccation preservation in mammal and human cells. This will probably require the use of other proteins, such as heat proteins or LEA proteins, or some completely unknown factor. If that is achieved then the day may come when dry preservation is possible at the organ or individual level.

The mechanism for the remarkable tolerance to extreme environments seen in anhydrobiosis is still little understood. If the mechanism through which biological structure is completely maintained even under intense environmental loads could be elucidated, the advance of living organisms into the ultimate environment of deep space would perhaps no longer be just a dream. It is fair to say that we have still taken only the first tentative steps toward understanding cryptobiosis, and there remains much to be studied in this exciting field.

13.4 CONCLUSION

Seki and Toyoshima³⁶ showed that tardigrades can survive even after exposure to high pressure of 600 MPa if the free water in their bodies has been reduced and they have been dried. Experiments with tardigrades have shown that water, which makes up over 70% of the mass of these organisms, plays an important role in preserving them over long periods, through periods of both animate and inanimate states. Moreover, it has been demonstrated using an extirpated rat isolated heart that resuscitation can be achieved after dry preservation for several days.⁴⁹ Water in the body includes free water and bound water, and it has been shown that by controlling the free water, organisms can dry out and cease biological phenomena semipermanently in an inanimate state (250 million years).⁵⁰

If water is then added, biological phenomena appear in a semibiology-like behavior (comparable to semiconductor behavior) from cryptobiosis.

In the 20th century, it developed into the physics, creating science, semiconductor.

In the 21st century, it begins the new age of semibiology, to develop into the biology.

Semibiology was born from the research of the tardigrade.

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14 Diversity of Lumbricid Earthworms in Temperate Agroecosystems

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14.1 INTRODUCTION

It is well known that earthworms (Annelida, Oligochaeta) have an important influence on organic matter decomposition, soil fertility, and soil structure. Their activity affects soil hydrology and stability, nutrient availability, and the activity of other soil organisms, thereby contributing to primary productivity in terrestrial ecosystems. The species found most commonly in temperate agroecosystems, particularly in North America, are members of the family Lumbricidae, a group with 16 genera and about 300 species worldwide (Lee 1985). Our discussion focuses on the taxonomic and functional diversity of lumbricid earthworms in agroecosystems, reports on how agricultural practices influence earthworm diversity, and finally provides information on how earthworm diversity may influence agroecosystem functions in temperate regions.

14.2 TAXONOMIC DIVERSITY OF LUMBRICID EARTHWORMS

Temperate agroecosystems of Europe commonly contain between 1 and 15 species of earthworms. Edwards (1983) reported that permanent pastures in the United Kingdom contain 12 species, on average, and cultivated soils have from four to six species. Many lumbricid species have been spread by Europeans to temperate regions of North America, New Zealand, and Australia, but the introduction of these earthworms was mostly accidental and some introduced species may have failed to survive. Baker (1998) reported that the number and species richness of earthworms in South Australia was low, with between 40 and 400 earthworms m⁻² and an average of 2.5 species in these temperate agroecosystems.

A review of selected papers since 1982 confirms that most cultivated soils in Europe have between four and six earthworm species, although agroecosystems under cereal production in Ireland contain up to 12 species (table 14.1). The dominant species in most temperate agroecosystems of Europe is *A. caliginosa*. Outside Europe, this species is often differentiated into the species *A. turgida*, *A. trapezoides*, and *A. tuberculata*. In temperate agroecosystems where earthworms were introduced, our literature review indicates that one to eight lumbricid species may be found (table 14.2). No introduced lumbricid species was dominant in the majority of agroecosystems, but this is not surprising given the global distribution of these studies. Other introduced earthworms and indigenous earthworms are sometimes found in temperate agroecosystems in Australia and the United States. Mele and Carter (1999a) found individuals from the family Acanthodrilidae (introduced species) and the family Megascolecidae (indigenous species) in the agroecosystems studied, but noted that lumbricid earthworms were numerically dominant. Similar findings were reported by Baker et al. (1993), Doube et al. (1994), Hubbard et al. (1999), and Parmelee et al. (1990).

Calculating diversity indices for earthworms based on species richness and the number of individuals of each species may not be appropriate for several reasons. It is generally impossible to identify all earthworms collected from field sites to the species level because more than 50% tend to be immature (Edwards and Bohlen 1996). Species diversity indices are based on the assumption of a log-normal distribution of species within a population, which may not be valid in species-poor populations (Magurran 1988). Finally, species diversity indices are weighted in favor of the numerically dominant species and do not readily distinguish rare or influential species, making it challenging to predict the effect of earthworm diversity on soil function. Generally, earthworm functional diversity is considered to be a more useful measure of earthworm biodiversity.

14.3 FUNCTIONAL DIVERSITY OF LUMBRICID EARTHWORMS

Lumbricid earthworms are often grouped into epigeic, anecic, and endogeic species based on feeding habits and life history strategies (Bouché 1977). Epigeic species, such as *Lumbricus festivus*, *L. rubellus*, *L. castaneus*, and *Dendrobaena rubida*, inhabit the litter layer of soils and feed primarily on surface residues. *L. terrestris* and *Aporrectodea longa* are considered to be anecic earthworms since they form deep permanent or semi-permanent vertical burrows in soil and come to the surface to feed on residues that they drag into their burrows. Endogeic species of earthworms include *A. caliginosa*, *A. tuberculata*, *A. trapezoides*, *A. rosea*, and *Octolasion lacteum*, which inhabit the mineral soil horizons and are thought to feed primarily on humified soil organic matter. Epigeic, anecic, and endogeic species interact with each other, with other soil organisms and with other functional domains in the soil such as the rhizosphere and the porosphere. The functional domain relating earthworm activity to soil processes is referred to as the drilosphere, and has been described by Lavelle (1988) and Brown et al. (2000).

The drilosphere effect for each functional group is dynamic, changing constantly in space and time. Functional groups exert their influence on soil processes in three major ways: (1) through the consumption, digestion, and mixing of organic substrates and soil particles, including redistribution of these materials in the soil profile; (2) by producing nutrient-rich, organo-mineral complexes (casts) that are deposited on the soil surface or within the profile; and (3) by creating macropores (horizontal and vertical burrows). The amount of casting and burrowing done by a functional group is proportional to the activity of individuals within the group, but these biogenic structures are persistent and can continue to affect soil processes even when earthworms are inactive or absent.

The dominant earthworm in European agroecosystems, *A. caliginosa*, is classified as an endogeic species (table 14.1). Endogeic earthworms such as *A. caliginosa*, *A. rosea*, *A. trapezoides*, *A. tuberculata*, *A. turgida*, and *O. tyrtaeum* are numerically dominant in other regions of the world (table 14.2). This suggests that endogeic earthworms may make more important contributions to the drilosphere effect in temperate agroecosystems than epigeic and anecic earthworms. In tropical regions, Fragoso et al. (1997) also found that endogeic species predominate in agroecosystems

TABLE 14.1
Population Density, Biomass, and Diversity of Indigenous Earthworms in Various Agroecosystems

Numbers (m ⁻²)	Biomass (g mg ⁻²) ^a	Number of species	Species identified ^b	Agroecosystem	Location	Reference
1–74	ND	4	<i>A. chlorotica</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>L. terrestris</i>	Cereal-legume rotation	Boigneville, France	Topoliantz et al. 2000
23–92	1.0–3.2 ^D	4	<i>A. caliginosa</i> , <i>L. castaneus</i> , <i>L. rubellus</i> , <i>L. terrestris</i>	Cereal	Siuntio, Finland	Nuutinen and Haukka 1990
19–116	18.4–84.3 ^W	4	<i>A. caliginosa</i> , <i>A. longa</i> , <i>L. rubellus</i> , <i>L. terrestris</i>	Cereal and ley	Uppsala, Sweden	Boström 1988
224–389	94.9–112.3 ^W	5	<i>A. caliginosa</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>L. rubellus</i> , <i>L. terrestris</i>	Cereal-legume-hay	Trondheim, Norway	Haraldsen et al. 1994
50–1200	8–360 ^W	5	<i>A. caliginosa</i> , <i>A. chlorotica</i> , <i>A. rosea</i> , <i>D. rubidus</i> , <i>L. rubellus</i>	Cereal-legume-hay	Surnadal, Norway	Hansen and Engelstad 1999
0–72	ND	5–6	<i>A. caliginosa</i> , <i>A. chlorotica</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>L. terrestris</i> , <i>O. cyaneum</i>	Cereal	Rothemstead, England	Edwards and Lofy 1982a, 1982b
530–776	94–114 ^W	6	<i>A. caliginosa</i> , <i>A. chlorotica</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>E. tetraedra</i> , <i>L. rubellus</i>	Cereal-legume rotation	Zeewolde, Netherlands	van der Werff et al. 1998
200–1160	15–200 ^W	9	<i>A. caliginosa</i> , <i>A. chlorotica</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>L. castaneus</i> , <i>L. festivus</i> , <i>L. terrestris</i> , <i>M. minuscula</i> , <i>S. mammalis</i>	Cereal-legume rotation	Kildaire County, Ireland	Schmidt and Curry 2001
25–700	25–90 ^W	12	<i>A. caliginosa</i> , <i>A. chlorotica</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>D. octaedra</i> , <i>D. rubidus</i> , <i>L. castaneus</i> , <i>L. festivus</i> , <i>L. rubellus</i> , <i>L. terrestris</i> , <i>O. cyaneum</i> , <i>S. mammalis</i>	Cereal	Kildaire County, Ireland	Curry et al. 1995

^a Biomass was measured on wet earthworms (W), dry earthworms (D) or not determined (ND).

^b The species in bold was the numerical dominant in each study.

TABLE 14.2
Population Density, Biomass, and Diversity of Indigenous Earthworms in Various Agroecosystems

Numbers (m ⁻²)	Biomass (g mg ⁻²) ^a	Number of species	Lumbricid species identified ^b	Agroecosystem	Location	Reference
0–28	ND	1	<i>A. trapezoides</i>	Cereal-legume rotation	Oregon, USA	Wuest 2001
8–110	0.64–7.26 ^D	1	<i>A. trapezoides</i>	Corn-soybean	Missouri, USA	Hubbard et al. 1999
121–425	34–120 ^w	1	<i>A. trapezoides</i>	Pasture-cereal	New South Wales, Australia	Doube et al. 1994
0–97	0–44 ^w	2	<i>A. turgida</i> , <i>L. terrestris</i>	Corn-soybean-cereal rotation	Ontario, Canada	Tomlin et al. 1995
0–892	0–30 ^d	4	<i>A. caliginosa</i> , <i>A. rosea</i> , <i>L. rubellus</i> , <i>O. tyrtaeum</i>	Cereal-soybean-sorghum rotation	Georgia, USA	Parnelee et al. 1990
8–1298	3.1–224.5 ^w	5	<i>A. rosea</i> , <i>A. turgida</i> , <i>L. rubellus</i> , <i>L. terrestris</i> , <i>O. lacteum</i>	Corn, soybean and pasture	Indiana, USA	MacKay and Kladvoko 1985
0–125	0–59 ^w	5	<i>A. longa</i> , <i>A. trapezoides</i> , <i>A. tuberculata</i> , <i>A. turgida</i> , <i>L. terrestris</i>	Corn-soybean-cereal rotation	Pennsylvania, USA	Werner and Dindal 1989
34–192	ND	5	<i>A. trapezoides</i> , <i>A. tuberculata</i> , <i>A. turgida</i> , <i>L. terrestris</i> , <i>O. tyrtaeum</i>	Corn	Iowa, USA	Berry and Karlen 1993
10–350	2–32 ^D	6	<i>A. caliginosa</i> , <i>A. trapezoides</i> , <i>A. tuberculata</i> , <i>L. rubellus</i> , <i>L. terrestris</i> , <i>O. tyrtaeum</i>	Corn	Ohio, USA	Bohlen et al. 1995
50–1200	ND	6	<i>A. caliginosa</i> , <i>A. longa</i> , <i>A. rosea</i> , <i>A. trapezoides</i> , <i>L. rubellus</i> , <i>O. cyaneum</i>	Cereal, pasture	Canterbury, New Zealand	Fraser and Piercy 1998
0–802	ND	6	<i>A. caliginosa</i> , <i>A. rosea</i> , <i>A. trapezoides</i> , <i>A. tuberculata</i> , <i>L. rubellus</i> , <i>O. cyaneum</i>	Cereal, pasture	New South Wales, Australia	Mele and Carter 1999a, 1999b

^a Biomass was measured on wet earthworms (W), dry earthworms (D) or not determined (ND).

^b The species in bold was the numerical dominant in each study.

compared to undisturbed savannah or forest ecosystems. Epigeic and anecic species were present at most study sites in Europe, but were absent from some temperate agroecosystems in other parts of the world (table 14.1 and table 14.2). The presence or absence of earthworm functional groups will probably affect soil processes such as nutrient and organic matter dynamics and pedogenesis; a more detailed discussion of these relationships is provided in section 14.5.

14.4 EFFECT OF AGRICULTURE ON EARTHWORM DIVERSITY

Many studies have determined how specific agricultural practices, such as tillage, cropping systems, residue management, and fertilizer and pesticide applications, influence the total number and biomass of earthworms present in agroecosystems. There are relatively few reports on how earthworm species diversity or functional diversity is affected by such agricultural practices.

14.4.1 TILLAGE

Tillage affects earthworm populations by changing the amount, quality and location of their food supply, and altering soil physical properties such as soil moisture and temperature. Earthworm populations are also susceptible to mechanical damage from tillage implements, and it has been estimated that rotary cultivation can reduce the biomass of earthworms in a field by up to 68% (Marinissen 1992; Boström 1995). Inversion of the top 10–20 cm of the soil profile during tillage exposes some endogeic earthworm species to avian predators (Giller et al. 1997). Generally, earthworm numbers are higher in no-tillage than conventional tillage agroecosystems (Edwards et al. 1995; Chan 2001; Kladivko 2001). Higher earthworm numbers and biomass in no-tillage agroecosystems have been attributed to more beneficial soil conditions, including the presence of surface litter, accumulation of soil organic matter, favorable temperature and moisture conditions, and a lack of disturbance. Yet, in some cases, earthworm numbers and biomass may be no different, or slightly lower in no-tillage than conventional-tillage agroecosystems (Kladivko et al. 1997). Such cases are often reported in the first years after pasture is plowed down and row crops grown, perhaps because the incorporation of pasture residues into the soil provides sufficient food for earthworms, especially endogeic and epigeic species such as *A. caliginosa*, *A. trapezoides*, and *D. rubidus* (Boström 1995; Nuutinen 1992; Doube et al. 1994).

There are few reports on how earthworm diversity changes in response to tillage, but anecic species such as *L. terrestris* and *A. longa* appear to be more susceptible to tillage than other functional groups. Edwards and Lofty (1982a) reported that anecic earthworms (*L. terrestris* and *A. longa*) declined by 58–85% and endogeic species (*A. caliginosa*, *A. chlorotica*, *A. rosea*, and *O. cyaneum*) were reduced by up to 45% in cereal plots that were plowed compared to those under no-till for 5 years. Similarly, Berry and Karlen (1990) found that *L. terrestris* populations declined by 71–83% while endogeic populations (*A. trapezoides*, *A. tuberculata*, and *A. turgida*) were between 29 and 55% lower in soils under corn production that were plowed for 12–13 years than no-till plots. Kladivko et al. (1997) studied earthworm populations in no-till and conventional tillage sites under corn–soybean rotations in Indiana and Illinois. Middens constructed by *L. terrestris* were observed in 3 of 14 conventional tillage fields and 9 of 14 no-till fields, suggesting that long-term tillage may cause local extinction of anecic earthworms (Kladivko et al. 1997).

In the short term, the effect of tillage on earthworm functional diversity may be more variable. Emmerling (2001) found that two to three of the endogeic earthworms found in reduced tillage (layer cultivation) systems were absent from cultivated soils, but an anecic species (*L. terrestris*) was found in both tillage systems. Boström (1995) reported that the number and biomass of anecic and epigeic earthworms (*A. longa*, *L. rubellus*, *L. terrestris*) was unchanged, and the population of endogeic earthworms (*A. caliginosa*) increased when lucerne ley were plowed for barley production.

The effects of tillage on earthworm diversity are variable and seem to be influenced by the intensity and frequency of tillage operations. In the longer term, changes to the soil environment

and mechanical damage of earthworms by tillage implements may cause greater reductions in anecic and epigeic populations than endogeic populations.

14.4.2 CROPPING SYSTEMS AND RESIDUE MANAGEMENT

Earthworm populations and diversity are generally higher in pastures than cropped soils, but whether it is the absence of plowing or the amount and quality of residue inputs to pastures that favors earthworm activity is not known. Fraser et al. (1996) surveyed agroecosystems in the Canterbury Plains, New Zealand, and found that the greater numbers, biomass, and diversity of earthworms was in soils under pasture production for more than 9 years, while the lowest numbers and diversity were in arable soils cultivated for the production of annual crops for >9 years (fig. 14.1). The five species present in long-term pastures included *A. caliginosa*, *A. trapezoides*, *L. rubellus*, *A. rosea*, and *O. cyaneum*, while soils under long-term arable cultivation generally contained only *A. caliginosa* and *A. trapezoides* (Fraser et al. 1996). These results suggest that long-term annual cropping can reduce or eliminate some earthworm species, although a few endogeic species will persist.

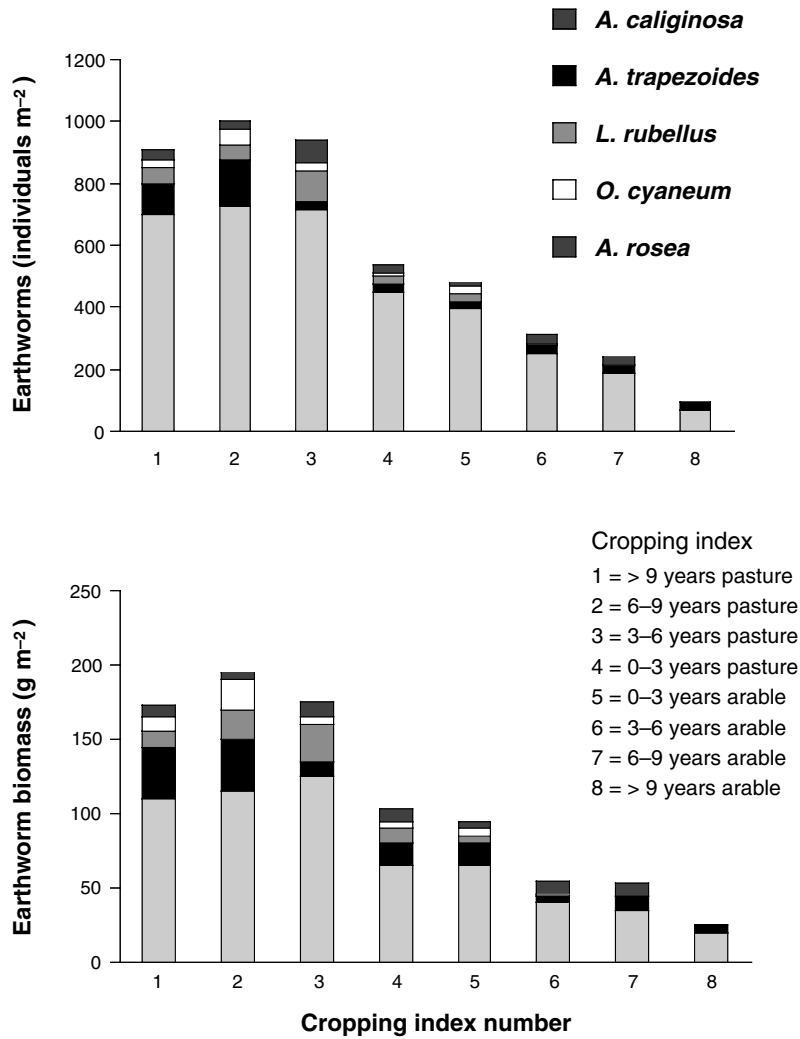


FIGURE 14.1 Effect of cropping intensity on the diversity, number (individuals m^{-2}) and biomass (g fresh weight m^{-2}) of earthworms in the Canterbury Plains, New Zealand (adapted from Fraser et al. 1996).

The type and sequence of crops grown in rotations can affect earthworm populations because these factors control the amount and quality of organic matter returned to the soil each year. Earthworms in temperate agroecosystems consume an estimated 2–15 Mg of organic residues ha⁻¹ each year (Shipitalo et al. 1988; Hendriksen 1991; Whalen and Parmelee 1999), indicating that the amount of residues required to sustain earthworm populations may be as high as the aboveground net primary production in some agroecosystems. Edwards (1983) noted that earthworm populations were higher in soils under cereal than soybean production because more residues remain after cereals are harvested and these residues are more resistant to decomposition than soybean residues. Other residues that may be beneficial for earthworms include straw and corn residues (Lofs-Holmin 1983; Werner and Dindal 1989).

In addition, including leguminous crops in the rotation can be beneficial for earthworm populations. The number and biomass of *A. trapezoides* were higher under a corn–soybean rotation than a corn–wheat rotation in no-tillage systems (Hubbard et al. 1999), suggesting that soybean residues are a better food source for earthworm populations than wheat residues. Similarly, cropping systems where cereals are undersown with legumes support higher earthworm numbers and biomass than those with monoculture cereals (Schmidt and Curry 2001; Schmidt et al. 2003; Fraser and Piercy 1998). This could be due to a higher input of organic matter or superior residue quality in cereal–legume intercropped systems than monoculture cereal systems. Also, soil moisture conditions may be more favorable to earthworms when an intercrop is present.

After harvest, crop residues may be left standing (stubble), mulched, burned, or incorporated by plowing. In the first few years after such management practices were implemented, the number and biomass of *A. trapezoides* was not much affected (Doubé et al. 1994; Chan and Heenan 1993). After 10 years, however, *A. trapezoides* and other earthworms were most numerous in agroecosystems where wheat straw was mulched and lowest in agroecosystems where it was incorporated by plowing (Mele and Carter 1999a). Yet, it is not known whether the decline in earthworm populations in the plowed treatment was due to differences in food availability or changes in the soil environment due to cultivation. Even when soils were maintained in bare fallow for 5 years, there was sufficient food to support the endogeic species *A. rosea*, *A. caliginosa*, and *A. chlorotica*, although populations were lower in the bare fallow (treated with herbicides and mechanical cultivation) than nearby plots seeded with mixed grasses (Auerswald et al. 1996). However, anecic and epigeic species such as *A. longa*, *L. rubellus*, *L. castaneus*, and *O. lacteum* were not present after 5 years of bare fallow (Auerswald et al. 1996).

It is clear that pasture systems can support a more diverse earthworm community than annually cropped soils, probably because these systems are tilled less frequently. The number of earthworms present in agroecosystems is influenced by the quantity and quality of residues returned to the soil, but whether earthworm diversity is influenced by crop residue management still needs to be investigated.

14.4.3 FERTILIZERS AND PESTICIDES

Inorganic and organic fertilizers containing the nutrients required for optimal crop yield are applied to most temperate agroecosystems. Earthworm populations are often greater in agroecosystems receiving inorganic fertilizers than those that receive no fertilizer, probably because fertilization increases net primary production and hence the quantity of residues returned to the soil (Edwards et al. 1995; Estevez et al. 1996). However, acidic soils receiving ammonia-based fertilizers tend to have lower earthworm populations than unfertilized soils, probably because such fertilizers lower soil pH to levels that are deleterious to earthworm survival (Ma et al. 1990). The application of NH₃-based manure slurry causes earthworm mortality, but populations generally recover within several months (Hansen and Engelstad 1999; Unwin and Lewis 1986).

The number and biomass of earthworms tends to be greater in soils amended with organic fertilizer (manure) than no fertilizer. Binet et al. (1997) and Andersen (1983) reported that there were generally more earthworms in plots amended with solid farmyard manure or liquid pig slurry than the control plots, which received no fertilizer. In addition, earthworm populations are often larger in cultivated agroecosystems receiving manure than inorganic fertilizer (Estevez et al. 1996; Werner and Dindal 1989; Whalen et al. 1998; van der Werff et al. 1998). Soil properties such as soil organic matter content, buffering capacity, aggregation, and water retention improve with manure applications (Schjonning et al. 1994; Haynes and Naidu 1998), which may make the soil environment more hospitable for earthworms. In addition, manure could be a food source for earthworms. However, earthworm populations did not increase when manure or inorganic fertilizers were applied to soils under a cereal–clover intercrop system or a grass–clover ley, suggesting that crop residues provided sufficient food for earthworms in these agroecosystems (Haraldsen et al. 1994; Schmidt et al. 2003).

Applying inorganic and organic fertilizers together may also be beneficial to earthworm populations. Edwards and Loft (1982b) found that continuous cereal plots receiving a combination of farmyard manure (35 Mg ha⁻¹) and inorganic fertilizer (96 kg N ha⁻¹) had more earthworms than plots receiving the same rate of farmyard manure alone or inorganic fertilizers (up to 192 kg N ha⁻¹). Yeates et al. (1997) reported that conventional grasslands, which receive NPK fertilizers and manure slurry every year, contained more earthworms than organic grasslands that were fertilized infrequently (once every 3 years or so) with manure.

Although the number of individuals is affected by fertilization, species richness and functional diversity tends to be similar among treatments (Edwards and Loft 1982b; Whalen et al. 1998; Schmidt et al. 2003). However, when earthworms were deliberately introduced to a polder soil, van der Werff et al. (1998) found that the average values of the Shannon-Weaver diversity index (H) for earthworms were significantly higher in soils receiving chicken manure ($H = 0.85 \pm 0.15$) than those receiving farmyard manure ($H = 0.41 \pm 0.11$) or no fertilizer ($H = 0.26 \pm 0.06$). Between 80 and 94% of the individuals found at this site were *A. caliginosa*. The value H was negatively correlated with the number of adult *A. caliginosa*, suggesting that the ability of other species were able to colonize and survive in soils dominated by *A. caliginosa* was affected by the type of manure applied.

Most herbicides are harmless or slightly toxic to earthworms, but plant residues killed by herbicides can be beneficial to earthworms since they serve as a food source (Mele and Carter 1999b; Edwards et al. 1995). Most pesticides used routinely on farms do not affect earthworm populations (Farenhorst et al. 2003; Heimbach 1997; Edwards and Bohlen 1996). Tarrant et al. (1997) found that variation in earthworm populations on British farms was related more to differences in soil texture and cultural history than a reduction in pesticide use.

Carbamate-based compounds, organophosphates, phorate, isozophos, chlorpyrifos, and thoprophos are toxic to earthworms (Edwards and Bohlen 1992). These broad-based insecticides are expected to be equally toxic to all earthworm species, although some species may have a great risk of coming into contact with pesticides due to their life history and feeding habits. Anecic and epigeic earthworms eat surface residues primarily; pesticides adhering to soil particles and organic matter at the soil surface may be inadvertently consumed by these earthworms. Potter et al. (1990) noted that some insecticides reduced earthworm populations, and toxicity was probably due to contact and ingestion of pesticides as earthworms foraged at or near the soil surface. Earthworms may also be exposed to pesticides that leach through the soil, and it is well known that the vertical burrows created by anecic earthworms are preferential flow pathways for water, nutrient, and pesticide movement through the soil profile (Edwards et al. 1993). Little information is available on how pesticides affect earthworm populations and diversity under field conditions and should be investigated further.

14.5 EARTHWORM DIVERSITY AND AGROECOSYSTEM FUNCTION

It is often difficult to develop predictive relationships between the diversity of soil food web organisms and the process rates crucial to agroecosystem function because these rates are strongly controlled by abiotic factors such as moisture, temperature, and human intervention (Bardgett 2002). Nevertheless, earthworms have been shown to influence many biological processes that are important for the functioning of temperate agroecosystems. Studies in Ohio demonstrated that earthworms alter soil aggregation, porosity, decomposition rates, nutrient mineralization, nitrogen leaching, and microbial activity in corn agroecosystems (Bohlen et al. 1997, 2002; Ketterings et al. 1997; Shuster et al. 2002, 2003). These findings are from field experiments where earthworm populations were manipulated through (1) earthworm additions (mostly endogeic and anecic species), (2) electroshocking, to reduce the number of earthworm present; or (3) undisturbed (ambient treatment). In New Zealand pastures, the inoculation of lumbricid earthworms has been shown to increase the rate of thatch removal and grass yield (Stockdill 1982). The addition of anecic (*L. terrestris* and *A. longa*) and endogeic (*A. caliginosa* and *A. chlorotica*) earthworms to no-till soils under cereal production increased root growth, compared to plots with ambient earthworm populations (Edwards and Lofty 1980).

It is likely that earthworm functional diversity influences the biological processes important for agroecosystem functioning in temperate regions. Epigeic, endogeic, and anecic species are expected to affect biological processes in distinct ways (table 14.3), but this needs to be verified under field conditions. Epigeic and anecic earthworms consume organic residues and deposit their casts on the soil surface, and are therefore expected to make a greater contribution to decomposition and aggregation processes at the soil surface than endogeic earthworms, since endogeic earthworms are most actively consuming organic substrates and depositing casts within the soil profile. Endogeic and anecic earthworms create semi-permanent burrows in the soil and therefore, are likely to have a greater impact on biopore formation than epigeic earthworms that live close to the soil surface and in the litter layer. Anecic earthworms are known to incorporate a greater proportion of surface residues into soil than epigeic and endogeic earthworms. All earthworms are known to stimulate nutrient mineralization through their interactions with soil microorganisms, but more of the nutrient

TABLE 14.3
The Expected Impact of Earthworm Functional Groups on Key Biological Processes in Agroecosystems

Biological process	Functional group		
	Epigeic	Endogeic	Anecic
Aggregation at the soil surface	High	Low	High
Aggregation within the soil profile	Low	High	Low
Formation of biopores	Low	High	High
Residue comminution	Low	Low	High
Decomposition—surface residues	High	Low	High
Decomposition—subsurface residues	Low	High	Low
Carbon sequestration	Low	High	Low
Nutrient mineralization	High	High	High
Nutrient loss	Low	Low	High
Microbial activity	High	High	High
Primary production	Low	High	High

losses due to earthworm activity is likely due to anecic earthworms; N loss through leaching and denitrification is greater in the burrows and middens formed by anecic earthworms like *L. terrestris* than the bulk soil (Edwards et al. 1993; Parkin and Berry 1994, 1999).

The impacts of earthworm functional groups on most of the biological processes identified in table 14.3 has been tested, but research on how earthworm functional diversity affects carbon sequestration and primary production in temperate agroecosystems should be verified under field conditions. We predict that endogeic earthworms will sequester more carbon than epigeic or anecic species because of the type of organic matter they consume (more humified substrates than fresh residues) and where they deposit their casts (within the soil profile, rather than at the soil surface). We also hypothesize that endogeic and anecic earthworms are more important for primary production than epigeic species, but they probably affect primary production in different ways. Endogeic earthworms likely increase the quantity of nutrients available for plant uptake when they are active in the vicinity of growing plant roots. Anecic earthworms may influence primary production by altering soil porosity, which would change water infiltration rates and rooting depth, and by accelerating the decomposition of surface residues.

Further research into earthworm diversity and agroecosystem function may permit us to develop predictive models of what functional groups (or species) should be present when temperate agroecosystems are managed in specific ways, and how the presence of these functional groups (or species) will affect processes such as organic matter and nutrient dynamics, soil structure, and crop production. Investigations of this nature would also permit us to determine the environmental impact and probability of survival of lumbricid earthworms introduced in temperate agroecosystems.

14.6 CONCLUSIONS AND FUTURE DIRECTIONS

Temperate agroecosystems may contain from 1 to 12 lumbricid earthworms, and lower species diversity is generally found in regions where these earthworms were introduced, either deliberately or accidentally, by European settlers. Due to similarities in their function, lumbricid earthworms are generally categorized as epigeic, endogeic, and anecic species. Agricultural practices, such as tillage operations, the selection of cropping systems and crop rotations, and the application of fertilizers and pesticides, affect the number and biomass of earthworms collected from agroecosystems. However, only tillage appears to alter earthworm functional diversity; with increasing tillage intensity, the proportion of epigeic and anecic species declines while the proportion of endogeics in the earthworm community remains the same or increases.

Future research is needed to determine how earthworm diversity is related to agroecosystem functioning. The epigeic, endogeic, and anecic species likely influence soil biological processes in distinct ways, but how these functional groups affect processes like soil quality and crop production in temperate agroecosystems, individually and together, is not well known. Such information is essential in establishing and maintaining earthworm communities that can enhance the functioning of temperate agroecosystems.

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15 Soil Enzymes: Spatial Distribution and Function in Agroecosystems

Ellen Kandeler and Richard P. Dick

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15.1 INTRODUCTION

Functioning of terrestrial ecosystems is dependent on biologically and biochemically mediated processes in soils. The soil biological community mediates critical ecosystem processes such as nutrient cycling, energy transformations, and degradation and cycling of complex organic compounds for which soil enzymes play a central role.

Enzymes are proteins that can catalyze reactions in an extracellular state in soils and aquatic systems. Extracellular enzymes may provide unique ecological functions for the soil microbial community by: (1) hydrolyzing substrates in pores that are not available to soil microorganisms (enzymes are ~100 times smaller than bacteria) followed by diffusion of the product back to the microorganisms and/or plant roots; and (2) transforming polymers into monomers or oligomers that can be taken up by membranes for intracellular metabolism (Quinquampoix et al. 2002).

In general, the biotic process of decomposition of organic matter and mineralization of nutrients can be studied at three levels of resolution (Sinsabaugh et al. 2002): (1) the molecular level that entails plant or organic matter fiber and specific enzymological characteristics of degradation; (2) the organismal level, which focuses on functional gene analyses, and regulation of enzyme expression and growth kinetics; and (3) the community level, which involves metabolism, microbial successions, and competition between microbial and faunal communities. These three levels must be integrated to fully understand microbial functions in soils. As an example, Sinsabaugh et al. (2002) explained decomposition as a complex successional/iterative loop where the

substrate controls the initial microbial community structure that then produces extracellular enzymes to degrade and modify the substrate and results in a new successional community or set of enzymes for evolving products or recalcitrant residues. Therefore, the activity of a range of enzymes can be used to study organic matter cycling of highly complex environments.

The objective of this chapter is to present an overview of soil enzymes relative to their (1) spatial distribution (micro- to landscape level) and (2) their ecological significance for microbial communities and ecosystem biogeochemical processes. This is important because it provides a basis for understanding microbial community functional diversity in agricultural soils. Reviews on basic soil enzymology include that of Skujinš (1967, 1976, 1978), Burns and Dick (2002), Kiss et al. (1975), Ladd (1985), Tabatabai (1994) and Beck and Beck (2000). Other useful information on soil enzymes related to soil and environmental quality and the plant–soil interface are presented by Dick and Tabatabai (1992), Dick (1994, 1997), and Dick et al. (1996).

15.2 SOURCES OF EXTRACELLULAR ENZYMES IN SOILS

Soil enzymes are largely of microbial origin but it is also possible that animals and plants may contribute enzymes to soils (Ladd 1978). It is difficult to conclusively discriminate between sources of enzymes in soils and thus evidence for the primary role of microbes as a source of soil enzymes results from indirect evidence (for a more detailed discussion, see the reviews of Ladd 1978; and Skujinš 1978). For example, it is expected that soil fauna are limited sources of enzyme activity in soils but there is relatively little information available to substantiate this. Earlier work provided evidence that earthworm (*Lumbricus terrestris*) casts have rates of high activity for a range of hydrolytic enzymes (Sharpley and Syers 1977; Park et al. 1990, 1992; Ross and Cairns 1982; Kiss 1957) and can also affect nitrogenase activity (Simek and Pizl 1989). It would be expected that these enzymes could be stabilized in the soil matrix as abiotic enzymes.

More recently, it has been shown that the type or quality of food fed to earthworms can affect enzyme activity levels. For example, Flegel and Schrader (2000) found that dehydrogenase, and acid and alkaline phosphomonoesterase activities in the casts of *Dendrobaena octaedra*, were affected by the food sources (various grasses, legumes, and woody materials). The activities of all 3 enzymes were significantly correlated with the organic C and the total N content of the casts. In another example, Mba (1997) showed that earthworms may be manipulated to excrete enzymes to mineralize P from organic inputs or rock phosphate to make more P available in P fixing soils. These results suggest earthworms may be managed to stimulate certain enzymes to enhance a biogeochemical process in agroecosystems to promote more sustainable agricultural methods and improve soil quality.

Plant roots can excrete extracellular enzymes (Rogers 1942; Rogers et al. 1942; Estermann and McLaren 1961; Dick and Tabatabai 1986). Rhizosphere soil increases enzymatic activity over nonrhizosphere soil for many enzymes (Skujinš 1978; Speir et al. 1980; Castellano and Dick 1991; Kandeler et al. 2002) but it is unclear whether this elevated activity is from enzymes released by roots or greater microbial biomass of the rhizosphere. It seems likely that roots stimulating microbial activity (Alexander 1977) would be a major factor explaining the rhizosphere effect on soil enzyme activity. Indirect evidence presented by Ladd (1978), Dick et al. (1983), and Nannipieri et al. (1983) suggests that enzymes from plant debris are rapidly decomposed and used for microbial growth in incubated soils.

The activity of approximately 100 enzymes has been identified in soils (Tabatabai and Dick 2002). Undoubtedly the number in soils is far greater but techniques for determining the presence or activity of other enzymes have not yet been developed for soils. The soil enzymes most often studied are oxidoreductases, transferases, and hydrolases. Those enzymes commonly extracted from soil, and their range of activities, are listed in table 15.1.

Enzymes can be constitutive and routinely produced by cells (e.g., urease). Whereas, others such as cellulase are adaptive or induced, and only expressed in the presence of a substrate or other

TABLE 15.1
Range of Activities and Reactions Catalyzed by Important Enzymes that Have Been Studied in Soils

Enzyme	Reaction	Range of Activity
Cellulase	Endohydrolysis of 1,4- β -glucosidic linkages in cellulose and lichenin, with release of β -glucose	0.4–80.0 μM glucose $\text{g}^{-1} \text{24 h}^{-1}$
β -Fructo-furanosidase (Invertase)	Hydrolysis of terminal nonreducing β -D-fructo-furanoside residues in β -fructofuranosides	0.61–130 μM glucose $\text{g}^{-1} \text{h}^{-1}$
β -Glucosidase	Hydrolysis of terminal, nonreducing β -D-glucose residues with release of β -D-glucose	0.09–405 μM <i>p</i> -nitrophenol $\text{g}^{-1} \text{h}^{-1}$
Proteinase	Hydrolysis of proteins to peptides and amino acids	0.5–2.7 μM tyrosine $\text{g}^{-1} \text{h}^{-1}$
Urease	Hydrolysis of urea to CO_2 and NH_4^+	0.14–14.3 μM N-NH_3 $\text{g}^{-1} \text{h}^{-1}$
Alkaline phosphatase	Orthophosphoric monoester + $\text{H}_2\text{O} \rightarrow$ an alcohol + orthophosphate	6.76–27.3 μM <i>p</i> -nitrophenol $\text{g}^{-1} \text{h}^{-1}$
Acid phosphatase	Orthophosphoric monoester + $\text{H}_2\text{O} \rightarrow$ an alcohol + orthophosphate	0.05–86.3 μM <i>p</i> -nitrophenol $\text{g}^{-1} \text{h}^{-1}$
Arylsulfatase	A phenol sulfate + $\text{H}_2\text{O} \rightarrow$ a phenol + sulfate	0.01–42.5 μM <i>p</i> -nitrophenol $\text{g}^{-1} \text{h}^{-1}$
Catalase	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$	61.2–73.9 μM O_2 $\text{g}^{-1} \text{24 h}^{-1}$

Adapted from Tabatabai and Fu (1992) and Nannipieri et al. (2002)

initiators or in the absence of an inhibitor. Dehydrogenases are components of the electron transport system of oxygen metabolism and they are often measured because they are not constitutive and also because they are found only in living systems (Speir and Ross 2002). Catalase activity is based on the rate of release of oxygen from added hydrogen peroxide or the recovery of hydrogen peroxide. Some hydrolases and transferases have been extensively studied because of their role in decomposition of various organic compounds and thus are important in nutrient cycling and formation of soil organic matter. These would include enzymes involved in the C cycle—xylanase, amylase, cellulase, lipase, glucosidases, and invertase; the nitrogen (N) cycle—proteases, amidases, urease, and deaminases; phosphorous cycle—phosphatases; and sulfur cycle—arylsulfatase. Lyase activity has been found in soils but relatively few studies have been conducted on this group of enzymes.

15.3 SPATIAL DISTRIBUTION OF ENZYMES IN SOILS

15.3.1 MICRO-SCALE DISTRIBUTION

The micro-scale (μm to mm), where enzymes are expressed at the molecular level and interact with the microbial habitat, is a fundamental process scale that ultimately controls biogeochemical cycles and microbial ecology. The location of enzymes in intracellular or extracellular locations and their interactions and spatial variation with the physical surroundings are the basis for the distribution of enzyme activities at higher spatial scales.

Soil enzymes can be classified according to their micro-scale location in the soil (Burns 1982). Figure 15.1 shows that soil enzymes can be associated viable cells or as extracellular enzymes. Biotic enzymes of proliferating viable cells can be classified into three categories according to their location: (1) intracellular in cell cytoplasm; (2) periplasmic space; and (3) outer cell surfaces. The remaining categories are broadly characterized as “abiotic,” a term first used by Skujinš (1976), which is derived from the Greek α -, the alpha privative meaning “removal or absence of a quality,” and the Greek suffix -bionic, meaning “having a form of life.”

Abiotic enzymes are those exclusive of live cells that include enzymes that are excreted by living cells during cell growth and division; attached to cell debris and dead cells; and leak into

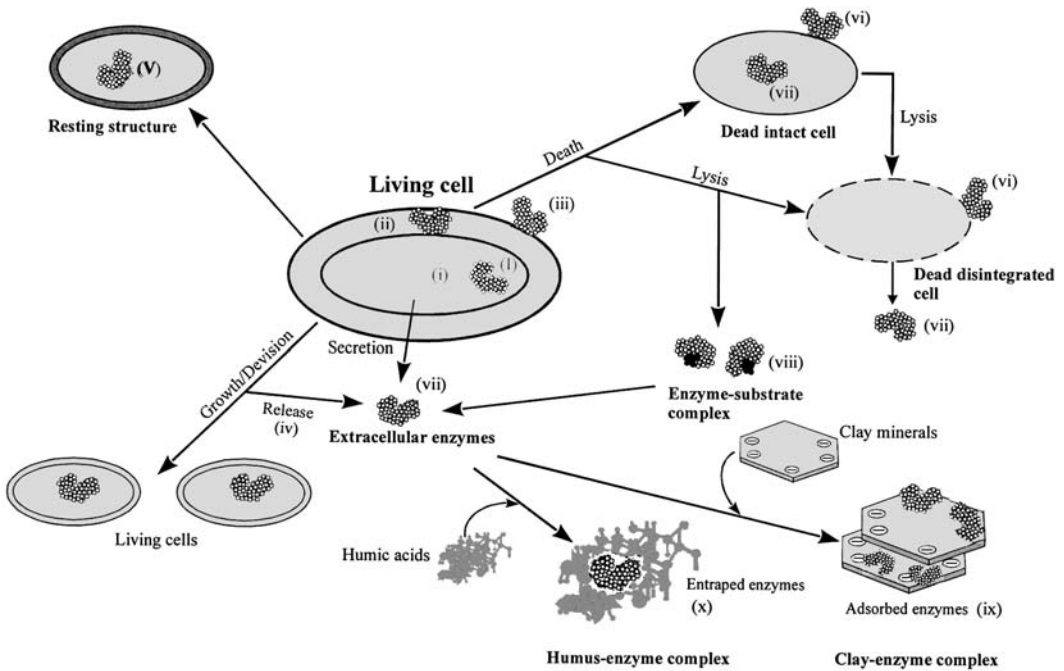


FIGURE 15.1 Small-scale distribution of soil enzymes (from Burns, 1982, adapted by Klose 2003)

- (i) Intracellular enzymes
- (ii) Periplasmic enzymes
- (iii) Enzymes attached to the outer surface of cell membranes
- (iv) Enzymes released during cell growth and division
- (v) Enzymes within nonproliferating cells (spores, cysts, seeds, endospores)
- (vi) Enzymes attached to dead cells and cell debris
- (vii) Enzymes leaking from intact cells or released from lysed cells
- (viii) Enzymes temporarily associated in enzyme-substrate complexes
- (ix) Enzymes adsorbed to surfaces of clay minerals
- (x) Enzymes complexed with humic colloids

soil solution from extant cells or lysed cells but whose original functional location was on or within the cell. Additionally, abiotic enzymes can exist as stabilized enzymes in two locations: (1) adsorbed to internal or external clay surfaces; and (2) complexed with humic colloids through adsorption, entrapment, or co-polymerization during humic matter genesis (Boyd and Mortland 1990; Quiquampoix et al. 2002).

The strong and largely irreversible adsorption of enzymes on mineral and organic phases of the soil has important consequences for their mobility, survival, and catalytic activity (Quiquampoix et al. 2002). The most well-known effect of adsorption of enzymes on negatively charged surfaces, such as clay, is a shift of their optimal catalytic activity toward a higher pH range due to both pH-dependent modification of conformation and pH-dependent orientation of the catalytic site of the enzyme. Therefore, the functionality and activity of enzymes in soils are closely controlled by their location relative to enzyme–substrate interactions occurring at pico- and nano-scales. Limited insights on enzyme location at these scales has been made by studying model humic-enzyme copolymers (Ruggiero et al. 1996), chemical extraction of enzymes from the soil matrix (Tabatabai and Fu 1992), and by modeling the immobilization of enzymes on various matrices (Quiquampoix et al. 2002). Generally, pico- and nano-scale studies have been performed using artificial systems (e.g., studies of adsorption of specific proteins onto clay particles) (Quiquampoix et al. 2002).

Recently there have been renewed attempts to separate microbial versus extracellular (stabilized in soil matrix) enzyme activities. Tabatabai and coworkers (Klose and Tabatabai 1999; Klose and Tabatabai 2002) and Renella et al. (2002) used chloroform fumigation to investigate this. Of the enzymes studied, arylsulfatase activity increased (Klose and Tabatabai 1999; Renella et al. 2002) with fumigation, whereas other enzymes such as glycosidases (Klose and Tabatabai 2002) and urease (Renella et al. 2002) activity decreased or remained nearly unchanged (β -glucosidase, acid and alkaline phosphatase, and protease) (Renella et al. 2002). A complicating finding for this approach is that the addition of protease inhibitors during the fumigation caused an increase in activity of urease and phosphatases (Renella et al. 2002), suggesting that some enzymes liberated during fumigation are subject to hydrolysis by proteases. From these results and since it is known from pure culture assays that microorganisms can hydrolyze the substrates for these enzymes, several things can be inferred: (1) arylsulfatase exists intracellularly because activity goes up with fumigation; (2) what is referred to as “extracellular” arylsulfatase and derived from the fumigation method is not solely abiotic activity but includes periplasmic and/or cell surface membrane enzymes because it is established that pure cultures can hydrolyze this ester sulfate bond; (3) only arylsulfatases outside the cytoplasmic membrane are involved in biogeochemical S cycling because lysing of cells causes activity to increase (i.e., suggests this substrate cannot penetrate cytoplasmic membrane unless it is lysed); and (4) the remaining enzymes that decrease or are unaffected by fumigation do not possess intracellular forms but may (likely?) have periplasmic/cell surface enzymes because we know pure cultures can hydrolyze these substrates.

Knight and Dick (2004) used an alternative approach to estimate abiotic activity; microwave irradiation (10 min in presence of 65 mL water) to denature microbial β -glucosidase. “Microbial β -glucosidase activity” was estimated by taking the difference between total activity and activity after microwave irradiation. The optimal irradiation input level was determined as the amount of energy that maximized soil sterilization (as determined by microbial staining and microscopy) while maintaining significant levels of enzyme activity. The biomass β -glucosidase activity correlated with microbial biomass C (chloroform fumigation/incubation) ($r=0.42$, $p<0.05$) but activity after microwave irradiation (abiotic) did not ($r=0.20^{NS}$), suggesting this approach is reasonable for estimating the activity of these two enzyme pools. This particular study demonstrated that the ability of β -glucosidase activity to detect treatment effects of long-term agricultural management was due more to abiotic activity than activity associated with the viable microbial pool. However, further, more detailed studies on a wider range of enzymes and soils is needed to determine the effectiveness of microwave irradiation in differentiating microbial versus abiotic activity.

Another approach for micro-scale studies is to characterize soil physical fractions by measuring activity on particle size fractions (sand, silt, and clay) (Jocteur Monrozier et al. 1991; Stemmer et al. 1998, 1999). Size fractionations release macro-organic matter and organic matter associated with mineral particles that form organo-mineral particles and micro-aggregates of contrasting structure, function, and stability (Tisdall and Oades 1980; Stemmer et al. 1998; Jocteur Monrozier et al. 1991; Lensi et al. 1995).

These fractions, due to mineralogy and organic matter chemistry, appear to affect the distribution and types of enzymes at this spatial level. Generally, soil organic matter chemistry ranges from minimally decomposed and coarse plant debris with a high C/N ratio to highly processed humic substances, which exist in organo-mineral complexes with a narrow C/N ratio associated with clay fractions.

The spatial and physical size distribution patterns of C sources control the location of microbial community members and associated enzymes. It is now evident that soil microorganisms as well as soil enzymes are heterogeneously distributed within the soil matrix. These individual properties of enzymes cause a preferential binding either to clay-humus-enzyme complexes or to particulate organic matter. For example, xylanase activity and fungal-derived phospholipid fatty acids showed a very close relationship to the particulate organic matter of the coarse sand fraction (Kandeler et

al. 1999a, 1999b, 1999c; 2000; 2001; Stemmer et al. 1999; Poll et al. 2003). This correspondence between the fungal enzyme producer with the enzyme and the substrate ensures efficient use of fungal substrates by the consumer (Kandeler et al. 2000). These results were also supported by the highly significant correlation between xylanase activity and the quality of organic matter (the signal of hemicelluloses measured by pyrolysis mass spectrometry; Leinweber et al., personal communication 2004).

It is well known that at the molecular level there is close attachment of cellulose-feeding bacteria (*Cytophaga*, *Cellulomonas*) with their substrate, without loss of the enzyme by diffusion into the soil solution (Alexander 1977). Therefore, enzymes involved in the hydrolysis of high molecular weight C substrates can tell us something about the location and perhaps the quality of soil organic matter in agricultural soils (Speir and Ross 2002). Other enzymes, invertase, alkaline phosphatase, protease, and urease, as well as bacterial biomass (as shown by PLFA and 16s rRNA gene fragments structural analysis; Kandeler et al. 2000) were more closely aligned with the finer soil fractions.

Clay-sized particles have a higher surface area than coarser fractions, which facilitates bacterial growth and attachment and protection of microorganisms and extracellular enzymes. Therefore, soil management (e.g., tillage and fertilizer treatments) will change enzyme activities associated with these fractions much slower than in coarser fractions (Kandeler et al. 1999a, 1999b, 1999c, 1999d, 1999e). Although these studies helped to understand the location and functioning of soil microorganisms at a small scale, the fractionation procedure destroys the natural microenvironment of particles and soil microorganisms (especially branching or hyphal organisms). This severe disadvantage can partly be overcome by applying techniques from physical and modeling approaches to provide insights into soil microbial processes in a three-dimensional soil ecosystem.

Some first attempts with these approaches are experimental designs focusing on the description and modeling of two-dimensional gradients of microbial activities that occur in the rhizo-, drilo- and detritussphere as well as preferential flow paths of soils (Kandeler et al. 1999b; Gaston and Locke 2002). Tarafdar and Jungk (1987), Tarafdar and Marschner (1994), Gahoonia and Nielsen (1991), and Kandeler et al. (2002) characterized rhizosphere soil with a root mat/soil interface separated by 53 μm nylon mesh in 0.1–0.2 mm increments for plant nutrient uptake and enzyme activities. The abundance of rhizosphere microorganisms and their activities decreased to levels similar to bulk soil within a distance of several mm from root surfaces. The distance of influence of the root on microbial parameters was related to the levels of easily degradable root exudates, mass flow, and diffusion distance of dissolved organic substrates used by soil microorganisms.

The small-scale heterogeneity of microbial abundance and activity is also due to the preferential flow of water in soils, which supplies the attached microorganisms with nutrients and substrates for their growth. For example, the flow of dissolved and particulate matter through preferential flow paths in soils was the reason for highly abundant bacterial cells in macropore channels (Vinther et al. 1999; Bundt et al. 2001b; Pankhurst et al. 2002). However, recent results indicated that there may only be a few communities that can utilize a broad spectrum of substrates as well as extremes in aerobic and anaerobic conditions that can profit preferential paths (Bundt et al. 2001a).

These recent results are beginning to provide evidence for an ecological role of extracellular enzymes, as first hypothesized by Burns (1982), in the functioning of microbial communities. Indeed, there may be species that may have evolved that do not expend energy for certain enzymes that are already found in soil solution or are intimately close to a cell but stabilized in organo-mineral complexes. Furthermore, there is preliminary evidence that enzymes associated with viable cells that are important to biogeochemical processes are located outside the cytoplasm in the periplasmic space or cell surface where substrates can reach the enzyme. However, considerably more research and new approaches are needed to fully elucidate the mechanisms for the role of soil enzymes in biogeochemical processes and microbial ecology. New molecular approaches that involve enzyme expression through RNA and proteomics hold potential to meet this need.

15.3.2 MESO-SCALE DISTRIBUTION

Meso-scale is the intermediate spatial pattern that ranges on the order of meters, which includes the distribution of soil enzyme activities both vertically (soil profile) and horizontally at the field scale under either agricultural or forest plots that receive relatively uniform long-term management. This scale is of particular importance because it has implications for ecosystem productivity, environmental quality, and assessment of management impacts on soil quality. Localized environmental pollution from accidental chemical spills and container leakage, mining, and industrial pollution occur at the meso-scale. Consequently, understanding the biogeochemical and enzyme-mediated processes at this scale is vital to guide sustainable management of ecosystems and bioremediation of pollution sites.

Meso-scale vertical distribution. The vertical distribution of enzyme activities are highest at the surface and decrease with depth, which has been shown for many enzymes and on every soil studied so far (Juma and Tabatabai 1978; Speir and Ross 1984; Dick 1984). Figure 15.2 is an example of the typical vertical distribution of activity from a forest soil.

Soil management can also affect the vertical distribution of enzyme activities. Figure 15.2 shows how compaction from forest skid roads during logging can have an effect well below the surface and, in this case, cause a reduction in enzyme activities at lower depths, which was attributed to reduced root growth in the lower depths (Dick et al. 1988b). Four years after the treatments were initiated, phosphatase activity was significantly decreased in compacted soil from 10 to 60-cm deep as compared to rehabilitated soil (fig. 15.2) and all enzymes tested (phosphatase, amidase, dehydrogenase, and arylsulfatase) were depressed from 41 to 75% in the 10- to 20-cm depth. Enzyme activities were negatively correlated with soil bulk density ($p < 0.05$).

In row crop agricultural systems, the level of disturbance from tillage can impact the vertical distribution of enzyme activities (Klein and Koths 1980; Dick 1984; Sequi et al. 1985; Angers et al. 1993). No-till systems have minimal disturbance, which results in significantly higher enzyme activities in the 0- to ~5-cm depth than soils that have regular conventional tillage (fig. 15.3 shows a typical example of this). Tillage tends to homogenize the organic matter inputs in the top 0- to 15- or 20-cm depths and results in a more uniform level of enzyme activity in this surface zone (fig. 15.3).

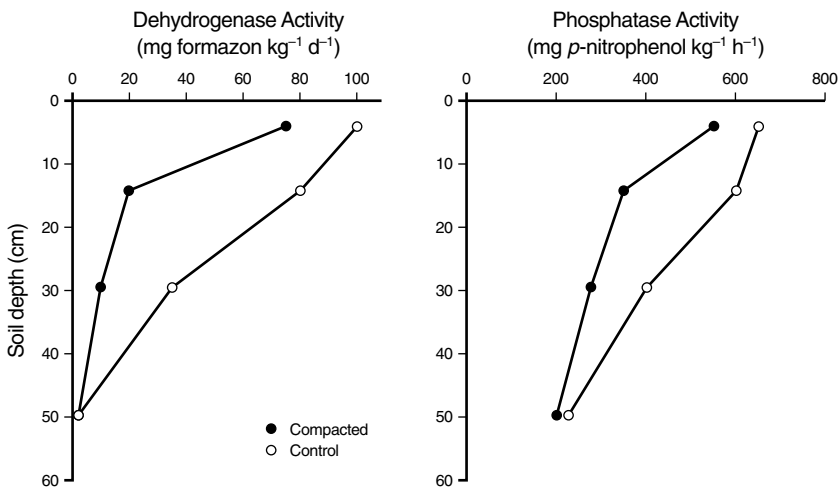


FIGURE 15.2 Distribution of dehydrogenase and phosphatase in the soil profile as affected by soil compaction in skid trails 4 years after logging compared to logged soil unaffected by skid tail equipment (adapted from Dick et al. 1988b).

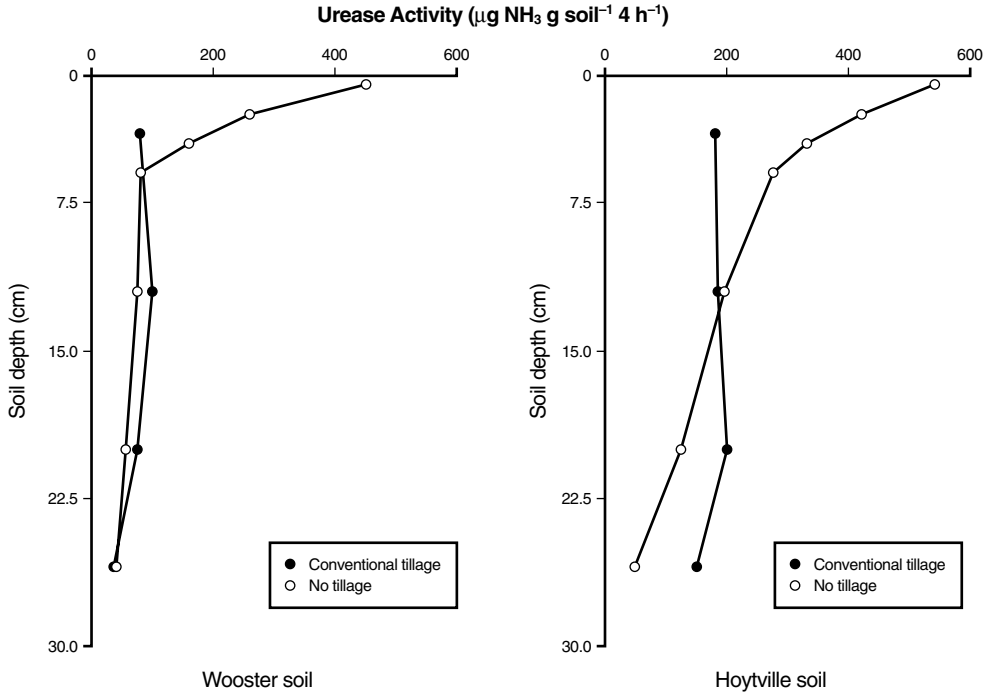


FIGURE 15.3 Distribution of urease activity in soil profiles after 18 years of varying tillage intensity under a corn-soybean rotation in Ohio (adapted from Dick 1984).

The vertical distribution varies as a function of soil type. This is illustrated in figure 15.3 where the Hoytville soil has higher levels of urease activity than the Wooster soil at all depths studied. This generally is related to soil genesis and texture. Soils with higher clay content and soils developed under grassland systems and over forest systems have higher levels of enzyme activities at lower depths.

These declines in activity with depth and the amount of activity at any given depth or differences among soil types are closely related with organic C and microbial biomass (see review by Speir and Ross 2002). First, the amount and quality of organic matter inputs control the microbial community size and structure (Schutter and Dick 2001). This of course explains why enzyme activities are normally highest at the surface where deposition of organic matter occurs. Organic matter inputs set in motion decomposition, which has a sequential, cascading effect on soil biology and food web processes that ultimately affect enzyme activity. Greater amounts of substrates and energy inputs supports a larger, more diverse, and active microbial community (Schutter and Dick 2001), which in turn increases enzyme expression. The variation in organic amendment chemistry affects the microbial response and the stimulation of a particular suite of enzymes.

The equilibrium activity level on mature undisturbed soils is not only a function of the microbial response to C inputs but also soil texture. This is because for a majority of enzymes (except dehydrogenase) a significant amount of the activity is associated with the abiotic fraction. Soils with higher clay content have a higher surface area and thus have greater potential to complex or adsorb and protect extracellular enzymes. At the same time, higher clay soils for the same reason can complex and protect more organic C compounds or colloids, which in turn can serve as entities that can complex and protect enzymes and also as habitats for greater microbial biomass/enzyme production.

Figure 15.2 shows that the vertical distribution pattern varies with enzyme type. This is likely a reflection of changes in the submicrobial population that is responsible for each enzyme. In turn

the type of substrates at a given depth would stimulate microbial production of specific enzymes to directly hydrolyze the substrate or its decomposition products.

Meso-scale horizontal distribution. The role of substrate inputs in affecting soil enzyme activities or dominance at landscape levels has been shown by investigations of disturbed landscapes that have been revegetated or allowed to return to native vegetation. Revegetation of soils after removal of top soil (Ross et al. 1982) or at a reclaimed coal strip mining site (Ross et al. 1992) in New Zealand showed that invertase and arylsulfatase were significantly correlated with phytomass yields within 2–3 years after establishing vegetation and reclamation treatments.

Studies of soil enzymes in disturbed landscapes that have been revegetated have provided a better understanding of the mechanisms of microbial community response during plant community succession (Pancholy and Rice 1973a, 1973b; Rice and Mallik 1977). These studies on succession from abandoned farmland to climax vegetation showed that in the early successional stages carbohydrases were highest (invertase, amylase, and cellulase), which corresponded to the pioneer weed species that dominated at this stage (these species have high cellulose and polysaccharide content). In later successional stages, carbohydrases declined when perennial grasses and woody species dominated, which was related to their higher lignin contents. Dehydrogenase and urease increased with plant community succession, which likely reflected the general buildup of the microbial biomass. These results were independent of soil organic matter, which was unaffected by the successional stage, showing the importance of the type of plant residues that are incorporated (substrate) and that soil enzyme activities are sensitive to changes in vegetation. These results were strikingly consistent across three different moisture regimes of plant community successions/ecosystems (varied in annual rainfall, 84 to 178 cm, and soil orders) (Pancholy and Rice 1973a, 1973b).

Field-scale investigations have shown that organic matter turnover rates, microbial biomass, and enzyme activities of soil samples vary with vegetation and soil types (Bonmati et al. 1991; Vaughan et al. 1994; Bahri and Berndtsson 1996; Stork and Dilly 1998). Relatively little is known about the topographic, pedogenic, soil mineralogy, and other properties that control microbial and enzyme distribution at landscape levels (Bergstrom et al. 1998; Stork and Dilly 1998; Wirth 1999) and the characterization of these interactions is essential to achieve a better understanding of complex ecosystem processes (Goovaerts 1998).

Research on spatial variability at the field or plot scale for soil properties has largely focused on soil chemical properties relating to soil testing for fertilizer management (Cahn et al. 1994; James and Wells 1990) and only rarely on spatial distribution of soil biochemical processes at the landscape levels (Smith et al. 1993). This latter scale is of particular importance for developing a soil quality indicator because the enzyme activities can vary more as a function of soil type than the differences caused by soil management. An example of this is the work by Bergstrom et al. (2000) who found significant effects of no-tillage on urease, glutaminase, β -glucosidase, and dehydrogenase, but that this effect was much smaller than topographic position or soil textural properties. In another study in agricultural soils, Bergstrom and Monreal (1998) showed that enzyme activities, one-third of the time over a growing season, were higher within the row than between rows of both soybean and corn. This was particularly true of corn, which was attributed to its larger rhizosphere that could provide greater C inputs to support the microbial community and stimulate enzyme production.

The meso-scale is an important landscape unit for guiding sustainable management of agricultural and forest ecosystems. It is at this scale that soil quality indicators can have practical applications and enzyme activities have the potential to be a subset of key measurements that can be used for this purpose. However, as we have presented above, meso-scale spatial variability of enzyme activities can be greater than soil management effects. Thus, more research is needed on spatial variability of enzyme activities on a wider range of soil types and ecosystems, and on the development of relative indexes or means to calibrate or interpret enzyme activities as soil quality indicators that are independent of soil type.

15.3.3 MACRO-LANDSCAPE DISTRIBUTION

The aggregation of micro- and meso-spatial soil biogeochemical processes to large terrestrial ecosystem scales has broad implications for the global ecology and environment. Specifically this scale affects climate, long-distance surficial movement of sediments across land forms, and functioning of aquatic systems. Environmental issues include global climate change relative to the partitioning C between CO₂ respiration back to the atmosphere or sequestering C in semi-permanent pools in soils during decomposition of photosynthetic derived plant C (Lal et al. 1996); nutrient loading of surface waters causing eutrophication; hypoxia or formation of the dead zone in the Gulf of Mexico from excess loss of fertilizer nutrients in the Mississippi River basin of the United States (Mitsch et al. 2001); reduction in stream quality due to sediment loading; and atmospheric pollution impacts on crop or forest productivity.

Tscherko and Kandeler (1999) investigated a subset of microbial factors at the meso-scale and found these were correlated and sensitive to site factors (land use, soil type, and pollution). These researchers went on to determine the importance of factors that influence microbial biomass, N-mineralization, and enzyme activities (xylanase, urease, phosphatase, arylsulfatase) on a large continental scale of Central Europe. In this case, 2,500 sampling points across different ecosystems were analyzed by conventional statistical procedures combined with fuzzy operations (Tscherko et al., personal communication 2004). In a multivariate, hierarchical view (cluster analysis with fuzzy numbers including all microbial variables), land management/disturbance was the strongest factor, while pollution was the weakest factor, governing the level of soil enzyme activities at the ecosystem level. Soil type turned out to be an important site factor as it summarizes climatic, topographical, and geological conditions, soil acidification, and vegetation influence (Tscherko 1999). A multivariate approach of fuzzy set operations revealed microbial biomass and arylsulfatase activity to be sensitive indicators for pollution across different ecosystems.

At the landscape scale, significant correlations have been found between organic C and enzyme activities (Dutzler-Franz 1977a, 1977b; Bergstrom et al. 1998; Wirth 1999). Based on this knowledge, models can be fitted to estimate and interpolate soil microbial activity values at unsampled locations. Geostatistical models (Warrick et al. 1986; Goovaerts 1998) have been used as a tool to estimate spatial dependencies and to predict soil attribute values. For example, protease activity of arable land in Austria was found to be spatially dependent to another sampling site at a distance of 40 m (Öhlinger et al. 1993). The range of spatial dependence was 20 m for phosphatase activity (Ap horizon) and 16 m for arylsulfatase along a slope of a Gray Brown Luvisol (Hapludalf) following harvest of soybean (Bergstrom et al. 1998), and no spatial dependency of dehydrogenase, urease, glutaminase, or β -glucosidase.

Decker et al. (1999) evaluated the patterns of variation of β -glucosidase, chitinase, phenol oxidase, and acid phosphatase in oak forest soils at spatial scales from 10s of km to <3 m. Spatial scale differences were detected at the regional, topographic, and single tree scale that were mainly due to strong nutrient availability gradients. The authors concluded that detrended correspondence analysis will help develop a scale-independent metric to be used for regional analyses in a geographic information system environment.

The macro-scale monitoring of soil enzyme activities can be used to integrate multiple soil microbial properties into a soil quality index. Halvorson et al. (1996) showed that soil enzyme activities, populations of microorganisms, pools of resources, and chemical soil properties covaried spatially and systematically across the landscape of over 220 sampling points in an agricultural field in southeastern Washington State. From this soil quality maps were made using kriging interpolation, providing the probability of a soil being of good or poor quality.

15.4 METHODS FOR STUDYING ENZYME ACTIVITIES IN SOILS

Standardized methods for a broad range of enzymes using colorimetric and fluorimetric techniques are available and have been described in detail by Tabatabai (1994), Alef and Nannipieri (1995),

Schinner et al. (1996), and Tabatabai and Dick (2002). Whereas the measurement of soil enzyme activities based on simple color development of the product has a long history and is widely reported in the literature, a fluorimetric-based enzyme assay was only recently developed (Marx et al. 2001; Vepsäläinen et al. 2001; Stemmer 2004). The fluorogenic enzyme assays are very sensitive and provide potential to detect enzyme activities of small sample sizes (e.g., micro-aggregates and rhizosphere samples) and/or low activity (samples of subsoil, peat, and soil solution).

Enzyme assays involve incubating soils with the appropriate substrate under strictly controlled conditions (e.g., temperature, pH, ionic strength, and time) and typically followed by extraction and colorimetric or fluorimetric determination of the product. An alternative approach is to analyze the substrate depletion. It is generally preferable to measure the product because measuring the change in substrate concentration is often small in comparison to the initial substrate concentration. Usually, an agent is used to inhibit microbial growth during the assay. Choice of inhibitor can affect enzyme activity results (Skujinš 1978). The most widely used reagent is toluene, which can inhibit some oxidoreductases but is without effect on most other enzymes (Skujinš 1967). Its usefulness is restricted to assays that last a few hours; longer assays may risk microbial proliferation (Tabatabai 1994). Thus, it is important to recognize that these assays are measuring potential enzyme activity because the incubations are done under optimal conditions that would not exist *in situ*.

A frequently determined enzyme activity is the oxidoreductase, dehydrogenase. This intracellular enzyme is involved in electron transport systems of oxygen metabolism and requires an intracellular environment of viable cells to express its activity. The catalysis of oxidation–reduction reactions are measured using a tetrazolium salt 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), which forms a reddish formazan product after an incubation period of several hours. In theory, dehydrogenase activity should be a good indicator for microbial activity, because these enzymes are bound to living and active cells. Recently, several shortcomings were published including interferences with heavy metal at high levels of pollution (mainly copper), catalysis of the assay substrate by extracellular phenol oxidase, or interferences with alternative electron acceptors like nitrate or humic acids (Tate 2002). Other oxidoreductase enzymes that have been used in soil studies are glucose oxidase, catalase, and peroxidase and phenol oxidase. Catalase activity was developed in the early 1970s and is based on the gas volumetric measurement of oxygen evolving after the addition of hydrogen peroxide to a buffered soil suspension (Beck 1971).

Another enzyme assay used to reflect general microbial activity in soil samples is based on the hydrolysis of fluorescein diacetate (FDA) (Schnürer and Rosswall 1982). FDA is hydrolyzed by a wide variety of enzymes including esterases, proteases, and lipases and is attributed to extra- and intracellular actions of enzymes (Guilbaut and Kramer 1964; Rotman and Papermaster 1966) and its hydrolysis has been found among a wide array of the primary decomposers, bacteria and fungi (Medzon and Brady 1969; Söderström 1977; Lundgren 1981). This has the potential to be an indicator of microbial activity because of the broad spectrum of enzymes that can perform this reaction. However, it does not always correlate well with other microbial properties such as microbial biomass or respiration (Bandick and Dick 1999). This may be due to the fact that some enzymes that perform this reaction have a significant level of abiotic enzymes in the soil matrix, which do not reflect current microbial properties.

The majority of enzyme assays that have been developed and used in soil studies are hydrolases because of their importance for specific reactions during organic matter decomposition and in C-, N-, P-, and S-cycles. For example, xylanase, cellulase, and invertase, three enzymes involved in C-cycling, can be measured by the release of the product after incubating soils with a buffered solution (pH 5.5) containing their corresponding substrates (xylan, carboxymethyl-cellulose, or sucrose). The released sugars cause the reduction of potassium hexacyanoferrate (III) in an alkaline solution. Reduced potassium hexacyanoferrate(II) reacts with ferric ammonium sulfate in an acid solution to form a complex of ferric hexacyanoferrate(II) (Prussian blue), which is determined colorimetrically (Schinner et al. 1996).

The activities of many other hydrolases—like phosphomonoesterase, phosphodiesterase, phosphotriesterase, or arylsulfatase—have been widely reported in the literature. These assays often use artificial substrates that do not occur naturally in soil, which typically are simple esters that combine the functional group of the substrate, for example, phosphate (for phosphatase) or sulfate with a chromophore, such as *p*-nitrophenol (PNP). PNP is desirable because it does not occur naturally and is highly extractable from soils with few interferences (except high organic matter soils where there can be extraction of soluble organics that also produce a yellow color and interfere with the colorimetric development of PNP). After a specific incubation period, a CaCl_2 -alkaline solution is added to stop the enzyme reaction and enables the extraction of PNP. The *p*-nitrophenol released is measured as a yellow compound under alkaline conditions.

Important enzymes in the N cycle are those that hydrolyze urea, proteins, and peptides and release ammonium from amino acids. Urease, which hydrolyzes urea into CO_2 , and ammonia makes N available to plants from fertilizer and animal wastes, and is, therefore, a frequently estimated enzyme. The urease assay measures released ammonia either by a colorimetric procedure or by steam distillation followed by a volumetric assay of ammonia (Kandeler and Gerber 1988). Amidase (acylamide amidohydrolase, EC 3.5.1.4) is an important enzyme in the N cycle that is involved in N mineralization releasing NH_4 from linear amides by acting on C-N bonds other than peptide bonds (Frankenberger and Tabatabai 1980). It is widely distributed in soils, plants (Frankenberger and Tabatabai 1982), yeast (Joshi and Handler 1962), and fungi (Hynes 1970, 1975). β -glucosaminidase activity has been shown to be related to N mineralization and releases NH_4 (Ekenler and Tabatabai 2002).

A range of alternative artificial substrates are available to assay extracellular enzymes in aquatic and terrestrial environments (Hoppe 1993; Freeman et al. 1995; Marx et al. 2001; Vepsäläinen et al. 2001; Stemmer et al. 2004). These substrates contain an artificial fluorescent molecule and one or more natural molecules (e.g., glucose, amino acids) and they are linked by a specific binding (e.g., peptide binding, ester binding). These substrates are conjugates of the highly fluorescent compounds 4-methylumbelliferone (MUB) and 7-amino-4-methyl coumarin (AMC) and are used to measure the activities of enzymes involved in C-cycling (β -D-glucosidase, β -D-galactosidase, β -cellobiase, β -xylosidase), N-cycling (leucine aminopeptidase, alanine amino peptidase, lysine-alanine aminopeptidase), P-cycling (acid phosphatase), and S-cycling (aryl sulfatase). The high sensitivity of these assays is the reason for increasing use of fluorogenic substrates in microbial ecology. One of the major disadvantages of these assays are the high background fluorescence of fluorogenic substrates due to the low purity of the commercial substrates, but a recently published method overcomes these problems by detecting the depletion of substrates by HPLC (high pressure liquid chromatography) (Stemmer 2004).

15.5 RESPONSE OF SOIL ENZYMES TO ENVIRONMENTAL CHANGE

There is interest in developing indicators of soil quality to reflect agricultural and forestry activity relative to important biogeochemical ecosystem processes (Doran and Jones 1996). Enzyme activities hold potential as a soil quality indicator because the assays often have relatively simple procedures, are integrative in nature, and are sensitive to land management (Dick 1997; Bandick and Dick 1999; Ndiaye et al. 2000). From the discussions in previous sections, it is clear that soil enzymes are the mediators and catalysts of important soil functions that include decomposition of organic inputs, transformation of native soil organic matter; release of inorganic nutrients for plant growth; N_2 fixation; detoxification of xenobiotics; and metabolism, nitrification, and denitrification. In essence, soil quality can be viewed as the ability of a soil to perform these functions, many of which are required for ecosystem functions. Enzyme activities have potential as an integrative index of the soil biological status or the ability of a soil to carry out discreet enzyme-catalyzed processes.

A few enzymes have potential as indicators of viable soil microbial biomass or activity. Most notable is dehydrogenase, but for the problems mentioned in the previous section it has not worked well as an indicator of microbial activity. Furthermore, enzymes that correlate closely with microbial activity may be less suited to predict long-term changes or trajectory in soil quality because they would reflect recent management or seasonal (climatic) effects that may be transitory.

The rationale for soil enzyme activity as a soil quality indicator is that enzyme activities are often closely related to important soil quality parameters such as organic matter, soil physical properties, and microbial activity or biomass (Dick 1997). Furthermore, they can begin to change much sooner (1–2 years) than other properties (e.g., soil organic C), thus providing an early indication of the trajectory of soil quality due to changes in soil management (Ndiaye et al. 2000). A conceptual model for enzyme assays as an integrative biological index is based on the observation that activity originates both from viable cells and abiotic components.

The abiotic component provides an indicator of semi-permanent changes in soil quality because such enzymes are likely complexed and protected in the soil humic- or clay-complexes. The interpretation of this from a soil quality perspective is that soil management that promotes stabilization of organic matter and associated structural properties (e.g., aggregation and porosity) would also promote stabilization of enzymes in the soil matrix. But since enzyme assays are much more sensitive than most other measures of soil organic matter, it can be viewed as an early and sensitive indicator of soil management. Soils that have been managed to promote soil quality (e.g., minimum tillage, organic amendments, crop rotations, etc.) should have higher biological activity, which would be reflected in greater enzyme production and the potential to stabilize and protect enzymes complexed in the soil matrix (i.e., through increased production of organic colloids and aggregation there is increased complexation and protection of enzymes). The following sections give examples showing the response of soil enzyme activities to man-made changes of agricultural ecosystems like climate change, agricultural management, and soil pollution.

Another potential advantage of some soil enzymes is that the assay can be run on air-dried soil samples. The effect of air-drying on enzyme activity varies with the enzyme and can cause an increase in activity, but for most enzymes, activity is reduced 20–60% (Bandick and Dick 1999). Screening of a range of enzymes has shown that although activity may decrease with air-drying, the relative change (or rank according to field management) in activity for some enzymes within the same soil type remains the same (Bandick and Dick 1999). Air-drying stabilizes enzyme activity, greatly facilitates sample handling, and allows for timely analysis (unlike most other microbial measures that must be done as soon as possible after sampling). Combining this with the relative simplicity of many enzyme assays makes it possible to run a large number of samples on a routine basis and would enable adoption by commercial soil testing laboratories.

Although oxidative enzymes would appear to be attractive for soil quality measurement because of their potential role in formation of organic matter, the assays generally only provide a qualitative measurement. This is because these assays cannot always be run under buffered pH conditions or there may be inorganic manganese and iron compounds, which can also catalyze the decomposition of the substrate, thus confounding the interpretation of the results.

In general, hydrolytic enzymes are good choices as soil quality indexes because it is likely that organic residue-decomposing organisms are the major contributors to soil enzyme activity (Speir and Ross 1976; Speir 1977). A number of assays have been developed that perform key reactions in the C, N, P, and S cycles. The most widely studied are those associated with enzymes that produce nutrient end-products that are important in plant nutrition (e.g., urease, phosphatases, and sulfatases). However, there is evidence that some of these can be confounded by long-term applications of fertilizers or liming. Phosphatase activity can be depressed by phosphate fertilizers and is also affected by pH, which is independent of other factors of soil quality such as organic matter content (see review by Dick 1994). Also, there is limited evidence that N fertilizers can have a similar effect on enzymes involved in the N cycle where the fertilizer has the chemical makeup of the product for a given enzyme (e.g., NH_4 for urease and amidase) (Dick et al. 1988b).

Therefore, as a general soil quality indicator, enzymes involved in the C cycle are likely to be better choices. For this reason, an important C cycle enzyme, β -glucosidase, which releases C for energy and is sensitive to soil management effects, has been recommended as a soil quality indicator (Bandick and Dick 1999; Monreal and Bergstrom 2000; Ndiaye et al. 2000). These same studies and that of Tscherko (1999) have shown arylsulfatase to be indicative of soil treatment effects and to be correlated to fungal biomass (R. P. Dick, personal communication).

15.5.1 ELEVATION OF CARBON DIOXIDE AND TRACE GAS EMISSIONS

Global concentration of atmospheric carbon dioxide (CO_2) has risen from about 280 ppm to 365 ppm during the last hundred years (IPCC 2001). Direct effects of elevated CO_2 on enzyme activities are unlikely, because CO_2 concentrations in soils are already 10–50 times higher than in the atmosphere (Lamborg et al. 1983). Nevertheless, considerable research has focused on whether soil microorganisms and soil enzyme activities are involved in the response of plant communities to increasing atmospheric CO_2 concentrations and play an important role in sequestering C in soils (Jones et al. 1998; Kampichler et al. 1998; Niklaus et al. 2001; Kandeler et al. 1998).

These studies provide evidence that changes in the activity of microbial communities due to environmental changes can have lasting effects on ecosystem functioning (Kampichler et al. 1998; Zak et al. 2000; Ebersberger et al. 2003). Several mechanisms have been suggested for how elevated atmospheric CO_2 might influence soil microbial communities as a secondary response to effects on plants.

First, elevated CO_2 stimulates plant photosynthesis, thus increasing assimilation, net primary productivity, and below-ground allocation of assimilates. Root biomass increases, the root-to-shoot ratio widens, more fine roots are produced, and their turnover is enhanced elevated CO_2 (Rogers et al. 1994). Second, plant chemistry of green leaves is altered with elevated CO_2 , with nitrogen (N) generally being depleted and C/N widens (Norby et al. 2001). This would be expected to affect the microbial communities as they adapt to lower N substrates.

Using a Mediterranean ecosystem model, elevation of atmospheric CO_2 was accompanied by an increase of dehydrogenase activity, which was attributed to a larger microbial biomass (Dhillon et al. 1996). Since xylanase, cellulase, and phosphatase activities as well as hyphal length of predominantly saprophytic fungi also increased, authors suggested that organic matter decomposition was stimulated and that additional storage of C in soils was not very probable. Contrasting results were obtained by Moscatelli et al. (2001) in a Mediterranean natural forest ecosystem. Microbial biomass, dehydrogenase, β -glucosidase, acid phosphatase, and protease activities responded only at the beginning of an open-top chamber elevated- CO_2 experiment. Several studies have shown that soil microorganisms utilize and respond to increased substrates at the soil surface if plants produce more biomass due to elevated CO_2 (Diaz et al. 1993; Zak et al. 1993; Rice et al. 1994; Dhillon 1996).

Another mechanism for effects of climate change on soil biology could be due to changes in soil moisture regimes. Elevated CO_2 reduces stomatal conductance of plants, boosting water-use efficiency. At a plant community level, this reduces transpiration and increases soil water content. Higher soil water contents under elevated CO_2 are a common observation in CO_2 enrichment studies in grasslands (Körner 2000). Soil matric potential is an important control of soil microbial activity, directly through osmosis, as well as indirectly by controlling the nutrient supply (Killham 1994). Up to a certain threshold, soil moisture promotes soil microbes and their activity.

In addition, global cycles of many trace gases like H_2 , CO , CH_4 , OCS , N_2O , and NO is mediated by microbial processes in soils. Conrad (1996) gave an excellent review showing the role of microorganisms and soil enzymes as controllers of atmospheric trace gas consumption and production. For example, gas consumption is due to H_2 oxidation mediated by abiotic soil enzymes, CO cooxidation by ammonium monooxygenase of nitrifying bacteria, and CH_4 oxidation is performed by methanotrophic bacteria using CH_4 for their growth. One further challenge for the future is to

integrate data on soil enzyme activities into models that explain trace gas emission from soils. One of the first attempts was made by Kang et al. (1998) who compared β -glucosidase activity and hydrochemistry to predict fluxes of methane and nitrous oxide in North Wales, UK. Whereas variation of methane emission could be explained by changes in SO_4^{2-} concentrations and temperature, nitrous oxide emission was controlled by β -glucosidase activity, nitrate content, and water table. This example clearly demonstrated that the measurement of soil enzyme activities as well as physicochemical properties of soils can be integrated into mathematical models, which can explain much of the variability of gas emissions.

15.5.2 SOIL MANAGEMENT

Field scale studies have shown that enzymes are differentially affected on a temporal basis due to soil management activities. These studies have shown that soil enzyme activities vary seasonally and over the long term, which has improved our understanding of the functioning and dynamics of soil (Dalal et al. 1991; Friedel et al. 1996; Salinas-Garcia et al. 1997; Bandick and Dick 1999; Ndiaye et al. 2000). In many cases, enzyme activities can be early predictors of the effects of soil management on soil quality and how rapid these changes would be expected to occur. For example, the response of alkaline phosphatase activities in the top soil of a chernozem was detectable within the second year after a reduced tillage system was put in place, whereas 4 years were required before significant effects were found for microbial biomass and N-mineralization (Kandeler et al. 1999e) (fig. 15.4). The long-term monitoring of alkaline phosphatase over a period of 14 years revealed that phosphatase activity depended highly on the tillage treatment and to a much lower extent on the year of sampling (Spiegel et al., unpublished data). In addition, this same study showed there was a slow response of substrate-induced respiration to the tillage treatments, which was attributed to differences in biomass C turnover rates. Ndiaye et al. (2000) showed that β -glucosidase and arylsulfatase activities can respond within 2–3 years after initiation of winter cover cropping compared to other physical and chemical (e.g., total organic C) properties, showing minimal measurable effects (Buller 1999).

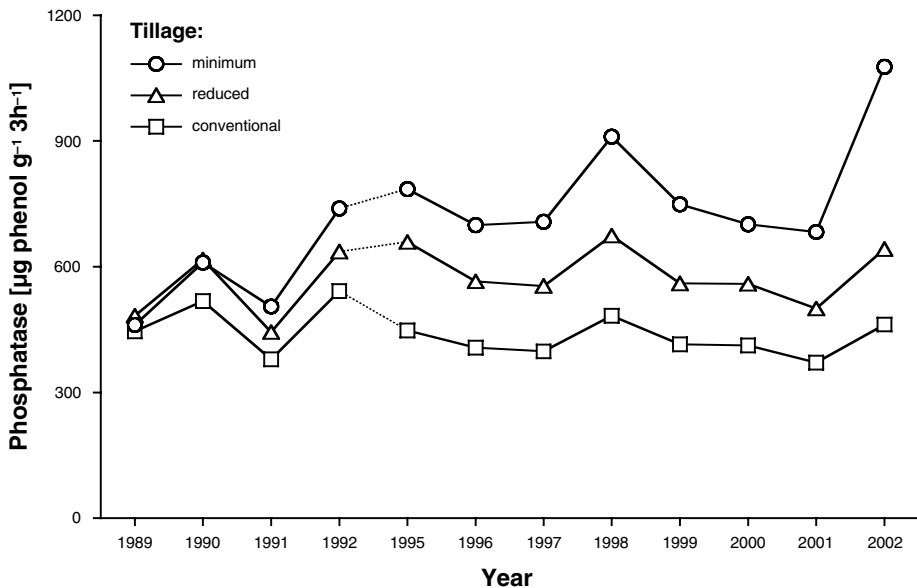


FIGURE 15.4 Temporal variation of alkaline phosphatase activity over a period of 14 years of the top soil (0–10 cm) of a fine-sandy loamy Haplic Chernozem at Fuchsenbigl (Lower Austria) (adapted from Kandeler et al. 1999e, and Spiegel et al. unpublished data)

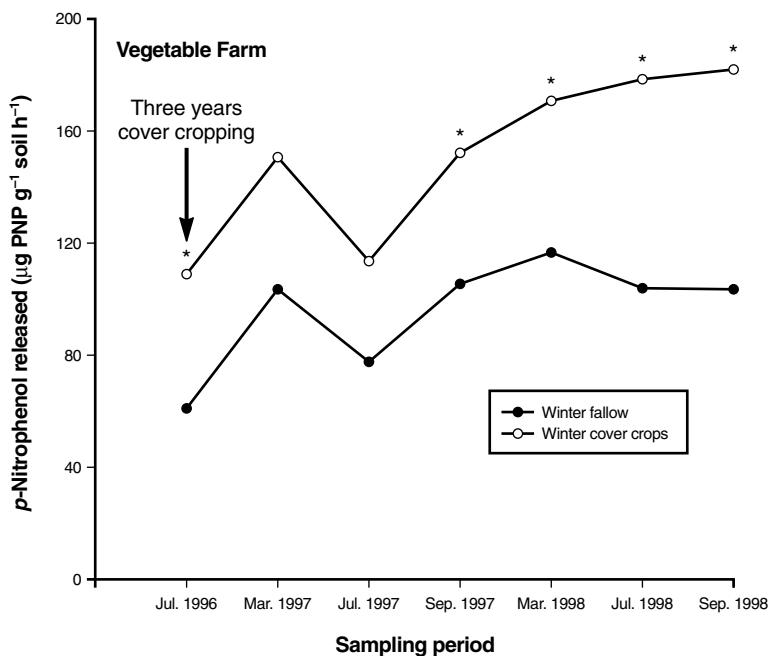


FIGURE 15.5 β -glucosidase activity after 3 years of cover cropping in a vegetable rotation in western Oregon (adapted from Ndiaye et al. 2000).

Many investigations have shown that organic amendments (plant material, animal residues, and sewage sludge) are rapidly decomposed in soil by microbial activity, releasing essential plant-viable forms of nutrients such as N, P, and S (Gianfreda and Bollag 1996). For example, high rates of cattle slurry application induced high enzyme activities of protease, deaminase, and urease and low root densities in a grassland soil profile (Orthic Luvisol, sandy silt, pH 6.6) (Kandeler et al., 1994). Since the roots were mainly located in the topsoil (0–10 cm) and N-mineralization was significantly enhanced at all soil depths in a slurry-amended grassland, it was concluded that nitrate leaching measured in lysimeters was due to N mineralization of different soil layers. In addition, crop rotations that maintain plant root activity most of the year and have biennial legume–green manure incorporations can improve soil chemical and microbiological properties within a relatively short time of 2 years (Miller and Dick 1995a, 1995b).

Numerous studies like those above have shown in side-by-side comparisons that enzyme activities are sensitive to soil management (see reviews by Dick 1994, 1997; Nannipieri et al. 2002). A few studies have shown that enzyme activities can be temporally sensitive within the first 2 years of changes in soil management, long before there are measurable differences in soil organic matter (Dick 1994; Ndiaye et al. 2000). Figure 15.5 shows such an early effect with a distinct separation after 3 years between soils managed with or without winter cover crops where there were no measurable changes in soil organic matter. However, not all enzymes are capable of distinguishing soil management effects and activities naturally vary as function of soil type (Bandick and Dick 1999). The choice of an enzyme assay for studying microbial ecology depends on the objectives and inferences that are desired from the proposed research.

15.6 CONCLUSIONS

Soil enzymes are central to ecosystem processes by catalyzing innumerable reactions in soils that have biogeochemical significance. Catalytic soil enzymes can exist internally or on surface membranes

of viable cells, be excreted into soil solution, or be complexed in the soil matrix or microbial debris. Extracellular enzymes may play an ecological role for some microbial community members by hydrolyzing substrates that are too large or insoluble for direct absorption by microbial cells.

Research on soil enzymes provides insights into biogeochemical cycling of C and other nutrients and on microbial community functions in space and time. A relatively small amount of any given enzyme can be directly extracted from soil; therefore enzymes are mainly studied by measuring activity. The activity of enzymes varies temporally (seasonal), which often corresponds to microbial community responses to the environment, vertically (decreasing from the surface), at micro-scales according to microbial community distribution, and at landscape level where soil type is a major controlling factor (particularly textural and organic matter).

Soil enzyme assays are emerging as technological tools for various applications in environmental and ecosystems management. Several enzymes have shown sensitivity in reflecting early changes (1–3 years) in soil quality due to soil management long before there are measurable changes in total organic C levels. This holds the potential to assist land managers in managing ecosystems for long-term sustainability. Enzyme assays can detect the level of degradation and recovery of soils in highly disturbed landscapes such as reclaimed strip mine landscapes. The bioavailability of certain heavy metals in soils can be reflected with enzyme activity measurements. Enzymes stabilized on colloid surfaces and incorporated into soils have been shown to degrade certain contaminants in soils.

There still is considerably more information needed to understand the ecology and function of extracellular enzymes in soils because of the diversity and complexity of the soil physical/chemical environment and microbial communities. In part because of this, utilization of enzyme technologies requires careful consideration for interpretation and application. This is particularly true for assessing soil quality where soil enzyme activities should be used in conjunction with other key soil measurements. Further research is needed to develop mechanisms for calibrating and interpreting soil enzyme technologies that are independent of soil type.

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16 Metabolic Diversity of Microorganisms in Agricultural Soils

Stefan Ratering, Gero Benckiser, and Sylvia Schnell

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16.1 INTRODUCTION

Natural ecosystems sustain soil fertility by an internal nutrient cycling. Climatic events are constraining the microfloral and faunal activities. From monocultured agricultural rotation systems, in contrast, large plantal portions are removed through harvesting and this nutrient removal differentiate them from natural successions. Farmers approach to compensate the removed nutrients by spreading mineral fertilizers, biotechnologically pretreated composts, sewage sludge, animal manures, and animal slurries (chapter 4 in this volume). These returned organic material added to the remaining plant residues differs in its chemical composition, and thus in its availability and biodegradability. Furthermore, agricultural soils are often treated with pesticides and are mechanically treated by ploughing, harrowing, or condensed by heavy machines. Agroecosystems, since several hundreds years under cultivation, seem, despite all these perturbations, enabled to cope with all these constraints. Biomass productions as high as those of natural ecosystem successions are documenting this (chapter 18 in this volume). Nevertheless the soil carbon content can decrease due to inappropriate land use and soil mismanagement practices, causing a decline in soil quality and a loss of C into the atmosphere (e.g., Blair [1], Lal [2], Wu et al. [3]).

The nutrient recycling in soils, especially that of carbon, is mainly processed by the microbial community. Mineralization is organized in a variety of respiration and fermentation processes, and ends with the release of carbon dioxide and/or CH₄ under anoxic conditions. Besides their degradation potential, soil microbial communities can also fix CO₂.

Hugh amounts of carbon are substructured in a vast variety of organic compounds that differ in turnover time. The chemical energy included is utilized by heterotrophic microbial and faunal communities that live on or inside soils, plants, or soil animals. Soil animals moving through and over the agricultural soil body spread the organic matter inclusively the incorporated microflora and thus add to the organic matter distribution through soil tilling practices and water movement. Moreover, it seems that intestination of bacteria by earthworms can activate these bacteria [4,5]. Niches are developing in which organic matter recycling can efficiently function.

This chapter tries to review the catabolic and secondary metabolism of soil microorganisms especially in agroecosystems.

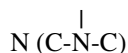
16.2 CARBON SUBSTRATE DIVERSITY IN AGRICULTURE

Germany produces annually about 7.5×10^6 tons of organic matter that are transformed into composts and an additional 26×10^7 tons of animal manures and about 37.4×10^7 m³ dry matter of animal slurries [6,7]. Considerable amounts of this organic matter are agriculturally recycled (chapter 4 in this volume). The introduced organic matter, together with the remaining plant residues after harvest, consists of a broad variability of organic compounds to be degraded in soils. Plant residues include cellulose (β -1,4-glucose polymer), starch (poly D-glucose chains of very different grade of polymerization), pectin (polymers of galacturon acids), hemicellulose (polymers of various hexoses, pentoses, and uronic acids), mannan (soluble polysaccharide of mannose), xylan (1,4- β -D-xylose polymer), fructane (β -2,1- or β -2,6-fructose polymer), proteins, lipids (fatty acid-glycerine-ester), waxes (fatty acid-alcohol-ester), chitin (β -1,4-N-acetylglucosamin polymer), and lignin (phenyl propane polymer). The soil-specific faunal and microbial community degrades these primary plant compounds to about 70% (w/w) within one year [6,8–11] in fertile temperate-zone soils. Jenkinson [8] found that the estimated half time of the original residues is 14–30 days and after the first year the residual C has a half-life of about 4 years. Additionally, plant compounds of minor quantity (approximate numbers of organochemical structures in parentheses) reach agricultural soils like alkaloids (6.500) and antibiotics, amines (100), amino acids (nonprotein, 400), cyanogenic glycosides (30), glucosinolates (75), simple phenols (200), flavonoids (4000), quinones (800) and poly acetylenes (650), terpenoids (mono-, di- tri- 4500) and other complex terpenoids like saponins (600), limonoids (100), curcubitacins (50), cardenolids (150), and carotenoids (500) [12].

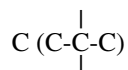
Besides the plant residues also organic compounds are returned to agricultural soils in the form of animal manures and composts. Compost material is degraded to about 15–50% (w/w) within one year and humus compounds have in aerobic soils mean residence times between 250–1,900 years [13–15]. From animal manures inside of animal housings volatile fatty acids (e.g., acetic, heptanoic, octanoic, and pelargonic acids), phenols and indoles (phenol, *p*-cresol, indole, skatole), methylamines, and other gases such as acetone disappear into the atmosphere [16]. Other volatile organic carbons released from soils are alcohols (e.g., ethanol, 2-methyl propan-1-ol, butan-1-ol), ketones (e.g., propane-2-one, 3-hydroxy butan-2-one) aldehydes (e.g., 2-methyl-butan-1-al, benzaldehyde), aromatics (e.g., benzene, trimethyl benzene), sulphides (dimethyl sulphide, dimethyl trisulphide, 2-methyl propylsulphide), and ethyl esters and methyl esters (e.g., 2-methyl butanoic acid, 3-methyl-butanoic acid). These metabolites are substrates that may additionally serve as signalling agents in the soil environment promoting, for example, mycorrhizal formation, suppressing fungal enzyme activities, or serving as a link between community members using detection and competition to advantage [17].

Proteineous, lipid-like, and aromatic polymer structures enriched in animal manures, composts, or peat material are often resistant to microbial degradation. Intrinsic limitations, which refer to the absence of biodegradative capacity, are lack of extracellular enzymes (for review, see Field [18]; chapter 15 in this volume), cell uptake mechanisms (large-sized molecules), or specific functionalities (lacking capability to insert molecular O₂ by oxygenases; anaerobes must introduce functional

groups with H_2O , CO_2 , or organic acids). Aromatic benzene and pyridine nuclei are reduced in their biodegradability when they are several-fold substituted by C-amine-, C-Cl-, C- SO_3H -, or C- NO_2 -. In contrast, hydroxyl and carboxyl groups facilitate degradation. Recalcitrant are also three-fold substituted



or fourfold-substituted



compounds. Unfavorable thermodynamic reactions, the steric hindrance due to branching of molecules or the inolarity of organic compounds, also contribute to soil recalcitrance. A low solubility in water means a high tendency to form micelles and an enhanced entrapment of pollutants inside of micropores [19] (chapter 3 in this volume). Soil homogenization, O_2 -bioavailability, easily degradable co-substrates, and a sufficient availability of electron acceptors (e.g., O_2 , NO_3^- , and Fe(III)) reduce the overall recalcitrance (for review, see Field [18]).

16.3 SUBSTRATE DEGRADATION

The lower the recalcitrance of organic matter, thus the easier a substrate is degradable, the greater is the number of competitors. Typical for many microbial environments including soils is a high fluctuation of substrate availability and often substrate limitation [20]. Accordingly, soil organisms react either with high growth rates but low substrate affinity (r-strategists) or with a highly efficient substrate utilization at low concentrations and low growth rates (K-strategists). However, in nature microorganisms grow mostly with a mixture of substrates [21]. The simultaneous utilization of mixtures of carbon substrates improves substrate capturing and thus lowers the threshold concentrations considerably, because mixed substrate growth allows microbes to still grow reasonably fast at minute concentrations of individual carbon compounds [22]. Mixed substrate growth confers not only competitive advantage to a microbial cell but also improves its metabolic flexibility toward changes in the environment (e.g., by shortening lags for induction of pollutant-degrading enzyme systems) (for review, see Kovarova and Egli [23]).

Co-substrates may also increase the capacity of microbial communities to degrade xenobiotic compounds [24] (for review, see Schink [25]). This type of degradation without substantial energy gain from the degraded xenobiotic compound is termed co-metabolism. Co-metabolism can be elucidated by employing substrates labeled with radioactive (e.g., ^{14}C) and/or heavy isotopes (e.g., ^{13}C or ^{15}N).

In the chemical literature currently over 10 million compounds with a surprising number of organic functional groups and structures are described. Organic compounds synthesized by microorganisms and plants are released under certain growth conditions or after death. Among the released compounds are antibiotics and plant-growth-promoting structures. The microbial soil diversity and thus the degradation capacity of all these compounds is influenced by environmental conditions and agricultural management [26–29, chapter 1].

Microorganisms since at least 3.6 billion years on earth have evolutionarily developed a great phylogentic and metabolic diversity. The high potential generation time of the microorganisms compared to higher organisms enables high evolutionary speed, resulting in development of further diversity. Examples for the microbial capacity of metabolism are given in the following sections.

16.3.1 DEGRADATION OF CARBOHYDRATE POLYMERS AND SUGARS

Cellulose, the most common organic substrate in nature, is a linear polymer of 10,000–15,000 glucose units linked by β -1,4 bonds and synthesized by plants and microorganisms (e.g., *Gluconacetobacter xylinum*). Each glucose residue is rotated by 180° relative to its neighbor molecules. The basic repeating unit is cellobiose. Numerous intramolecular and intermolecular hydrogen bonds make the fibrils so rigid and insoluble that it confers high tensile strength.

Structural analogues of cellulose are homo- or heteropolymer *hemicelluloses*. They consist largely of β -glucosidal (β -1,4, β -1,6, β -1,3, etc.) linked D-xylose, D- mannose, L-arabinose, D-glucose, D-galactose, and D-glucuronic acid. Hemicelluloses with β -1,4-D-glucose and D-mannose are *glucmannans*, β -1,3-*xylans* are hemicelluloses with a xylose backbone and glucuronic acid-arabinose side chains esterified to aromatic (phenolic) acids. Hemicelluloses probably are the second most important carbohydrate source in nature.

Other carbohydrates are *pectin*, α -(1,4)-linked sugar acid polysaccharides of the plant cell wall matrix (galacturonans), and *chitin*. Chitin occurs as cell walls in fungi, algae, and some plants, and as exoskeletons of invertebrates and represents the third most important natural carbohydrate source consisting of (1-4)- β -linked N-acetyl-glucosamine.

The most common storage material of plants is *starch*, a branched α -D-glucose composed of α -1,4-linked backbone amylose with varying amounts of α -1,6-linked amylopectin branches formed out of a few hundred to thousand glucose units per branch. Inulin (1,2- β -D-fructan), dextran (1,6- α -D-glucan), hyaluronate (β -1,5-linked N-acetyl- β -D-glucosamin and D-glucuronate), chondroitin (chondritin 4- and 6-sulfates), dermatan (1,4- β -N-acetyl-galactosamine), levans (2,6- β -fructose), agarose (1,3- β -galactose), or xanthan finally complete the natural sugar polymer spectrum.

These sugar polymers are degraded by many soil organisms and by microorganisms in animal gut systems. Degradation proceeds aerobically to CO₂ and anaerobically to CO₂ and CH₄. The activity of soil animals (chapters 11–14 in this volume) and ruminants (chapter 18 in this volume) greatly enhance the carbohydrate degradation by decreasing mechanically the particle size that allows easier access of microbes to the substrates. Microorganisms depolymerize the polymers outside of their cells by exoenzymes (chapter 15 in this volume). Extra cellular bacterial and fungal cellulases (exoglucanases) are needed to degrade the amorphous and crystalline regions of cellulose (chapter 15 in this volume), whereby the crystalline structures are more resistant to an enzymatic hydrolysis. The diversity of cellulolytic bacteria in one environment (e.g., in agricultural encatchment) showed high diversity including species of *Cellulomonas*, *Paenibacillus*, and *Streptomyces* [30]. A few examples for aerobic cellulolytic bacteria are various species of *Cellulomonas* sp. [31], *Isotericola variabilis* (formerly *Cellulosimicrobium variabile*) [32], *Cytophaga* and *Sporocytophaga* [33], *Thermoactinomyces* [34], *Thermomonospora* [35], *Streptomyces* [30], and *Bacillus* sp. [30,36]. Anaerobic soil microorganisms degrade about 5–10% of the cellulose. Important anaerobic species for cellulose degradation belong to the families of *Clostridia* (e.g., *Clostridium thermocellum*, *C. phytofermentans* [37], *C. ulginosum* [38], *C. acidisoli* [39], *C. hungatei* [40]). Other anaerobic cellulolytic bacteria, for example, found in paddy soils, belong to the *Verrucomicrobia* division, the *Cytophaga*–*Flavobacterium*–*Bacteroides* group, or the *Bacilliaceae* [41]. The species of the rumen microflora and fauna digest the cellulose completely anaerobically by having cellulosomes consisting of more than a dozen peptides and glycoproteins (for review, see Bayer et al. [42]) are listed by Leschine [43] and chapter 18 in this volume. Other soil bacteria profit from free sugars generated by the cellulolase activity of other microorganisms. Many soil bacteria grow with sugars if available, for example, *Burkholderia* spp., *Frateruria aurantia* [44], *Arthrobacter* spp. [45], *Acinetobacter* spp. [46], *Flavobacterium* spp. [47], *Massilia* spp., *Pedobacter* spp. and *Pseudomonas* spp. [48], *Aeromonas* sp. [49], *Sporomusa silvatica* [50], *Lactovum miscens* [51], *Pantoea punctata* and *Pantoea terrea* [52], and *Spirilliplanes yamanashiensis* [53].

Glucoamylases, β -, α -amylases, and amylo-1,6-glucosidases, another example of extracellular enzymes, split the helical starch structure into the single glucose units. After extracellular depolymerization the various sugars (C6, C5) can be taken up by microbial cells. The degradation of the

polymers is usually the rate-limiting step. The concentration of free sugar monomers in soil is in the μM range [4] because a broad microbial community takes it up fast and efficiently.

The initial steps are the conversion of glucose, fructose, mannose, xylose, glucans, fructans, and N-acetylglucosamine into fructose-6-phosphate. Additional reactions remove substituents such as $-\text{NH}_2$, $-\text{OCH}_3$, or $\text{OOC}-\text{CH}_3$ groups. Fructose-6-phosphate (C6) degradation proceeds according to the Embden–Meyerhof–Parnas (glycolytic) pathway in a stepwise sequence of reactions to the C3-intermediate pyruvate. The Embden–Meyerhof–Parnas (glycolytic) pathway is most common under aerobic and fermenting bacteria. Alternative or additional pathways for sugar degradation in microorganisms are the Entner–Doudoroff pathway and the pentose phosphate cycle. The Entner–Doudoroff pathway (for review, see Conway [54]) can be found in different phylogenetically distinct groups of bacteria like in the *Pseudomonas* species (for review, see Latour and Lemanceau [55]), in *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, *Paracoccus versutus* [56], *Shewanella oneidensis* (formerly *S. putrefaciens* MR1 [57]), and *Zymononas mobilis* [58], Galacturone, D-galacturonate, and uronic acid degradation proceeds according to the Entner–Doudoroff pathway. In most saccharolytic archaea the Entner–Doudoroff pathway is modified in a way that none of the intermediates are phosphorylated [59,60]. Halophilic archaea such as *Haloferax* and *Halococcus* use a partially nonphosphorylated Enter–Doudoroff pathway [59]. Also, modified Embden–Meyerhof–Parnas pathway was found among hyperthermophilic archaea [61,62] and these appear to operate in addition to nonphosphorylated Enter–Doudoroff pathway. The Pentose phosphate cycle is used, for example, by *Gluconobacter oxidans*, *Thiobacillus novellas*, and *Brucella abortus*. However, many bacteria degrade glucose and pentose partly via this pathway, whereas the greater part is degraded via one of the other pathways. The product of the pentose phosphate pathway is glyceraldehydes, which is further degraded to pyruvate in the same manner as via the Embden–Meyerhoff–Parnas pathway. Besides its function in degradation the pentose phosphate pathway has an important role in anabolism, especially in the synthesis of nucleotides.

Pyruvate as a common product of sugar metabolism is further degraded in various fermentation and respiration processes. For many respiratory processes pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase multi enzyme complex to acetyl–CoA, which enters either the tricarboxylic–acid cycle (Krebs-cycle) or the acetyl–CoA pathway with CO-dehydrogenase as the key enzyme. In fermenting bacteria other enzymes metabolizing pyruvate are common, like (1) pyruvate ferredoxin oxidoreductase yielding acetyl–CoA, reduced ferredoxin, and CO_2 , (2) pyruvate–formate–lyase in formate excreting enterobacteria; (3) or the pyruvate decarboxylase yielding acetaldehyde and CO_2 . Many fermentation processes, starting with sugars or pyruvate, are listed below:

1. During ethanol fermentation acetaldehyd is decarboxylated to ethanol.
2. In the homolactate fermentation, carried out by most of the *Lactobacillus*, *Lactococcus*, and *Streptococcus* species, pyruvate is reduced to lactate.
3. In heterolactate fermentation acetaldehyde is partly reduced to ethanol or oxidized to acetate by *Leuconostoc* sp. and *Lactobacillus* sp. and depending on the sugar utilized in *Streptococcus* species.
4. A fourth fermentation strategy where glucose is finally converted into butyrate and acetate, ethanol or acetone is the butyrate fermentation by *Clostridium pasteurianums*, *Eubacterium* sp., or *Fusobacterium* sp.
5. At pH values below 5 *Clostridium acetobutylicum* forms ethanol, acetone, 2-propanol, and 1-butanol as products.
6. Enteric bacteria and bacilli perform a complex fermentation, leading to various concentrations of lactate, formate, acetate, ethanol, and succinate.
7. *Propionibacterium* sp., *Clostridium propionicum*, and *Megasphera elsdenii* utilize the fermentation product lactate to produce propionate by methylmalonyl-CoA pyruvate transcarboxylase.

Some of the fermentation products can be further degraded by secondary fermenters in close metabolic association with syntrophic partner bacteria. The thermodynamic situation of syntrophic fermentation (for review, see Schink [25]) allows the process only in close exchange of mainly hydrogen (or formate) to hydrogen-utilizing microorganisms, which lowers the partial pressure of hydrogen to rather low values. A classical anaerobic syntrophic degradation example of fermentation products is the conversion of butyrate, propionate, or branched-chain fatty acids by *Syntrophomonas*, *Syntrophospora*, *Clostridium*, or *Desulfovibrio* in the presence of hydrogenotrophic methanogenes to acetate, CO₂, and CH₄. Primary alcohols like ethanol can be fermented syntrophically to acetate and methane. The hydrogen partial pressure has to be kept low (10⁻⁵ Pa, to 10⁻³ Pa depending on the substrate) by a hydrogen-scavenging partner for thermodynamic reasons (for review, see Schink [63]). As H₂ utilizing syntrophic partners methanogenic archaea, sulfate or sulfur reducers or acetogenes can function [63].

Whole genome sequencing revealed that quite a few microorganisms seem to lack enzymes at critical reaction steps of glycolysis, pentose phosphate pathway, and tricarboxylic acid in its understood way [64]. These microorganisms possibly use alternative enzymes or have modified at gene or function levels in order to maintain the pathway functionality [64].

16.3.2 DEGRADATION OF LIPIDS AND HYDROCARBONS

Lipids are membrane constituents of all organisms and as triglycerol fatty acid ester storage material in eukaryotes. Many growing microorganisms produce glycolipids. Glycolipid-microdroplets function as surfactants at polar–apolar interfaces. Lipases hydrolyze lipids into di- and monoglycerides, glycerol, and fatty acids. Fatty acids are further degraded via β -oxidation.

Hydrocarbons (linear or cyclic) are widely distributed compounds in environments and are produced by animals (e.g., insect secretion), plants (e.g., waxes of leaves), or microorganisms. Furthermore, they are generated by geochemical transformation processes leading to the formation of petroleum. Many man-made hydrocarbons starting from petroleum-like pyrethrin insecticides and pesticides (e.g., hexadecyclopropane carboxylate) are released in the environment. Hydrocarbons are classified according to their bonding features into saturated compounds (alkanes), compounds containing C=C double bonds (alkenes), compounds with C $\equiv$ C triple-bonds (alkynes), and the aromatic hydrocarbons (mono- and polycyclic). With the exception of the aromatic hydrocarbons the hydrocarbons can be straight-chain, branched chain, and cyclic (alicyclic).

Degradation of hydrocarbons under oxic conditions has been well known for several decades. The grade of susceptibility to biodegradability are in the following order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes [65]. Degradation pathways of simple hydrocarbons can be summarized as a set of “rules” [66].

- 1) Simple ketones are hydroxylated and/or oxidized to alicyclic alcohols and hydrocarbons and then attacked by mono-oxygenases that form the corresponding lactones. Ring cleavage occurs upon hydrolysis of the lactone and subsequent oxidation of the acid. Products formed are intermediates of the central metabolic pathways.
- 2) Alicyclic ketones with hydroxyl and methyl substituent groups are also degraded by the action of lactone-forming mono-oxygens. In addition, there is evidence that nonoxygenative cleavage reactions may occur in a minority of cases.
- 3) Cyloalkylic acids are degraded by one of a number of alternative routes, most commonly the ring is cleaved by β -oxidation. An alternative, restricted to the cyclohexane ring, involves hydroxylation at C₄, followed by dehydrogenation and aromatization of the ring to yield p-hydroxybenzoate, which is further metabolized by well-known routes involving the participation of oxygenases.
- 4) Substituted cyclopropanes are, on the basis of existing evidence, atypical. This is probably associated with the properties of the highly strained ring. Cyclopropane, for example, is unstable and ring-opens spontaneously in solution. Cyclopropane carboxylic acid and 1'-aminocyclopropane-1'-carboxylic acid are cleaved by mechanisms that are not yet well understood but that may not be generally applicable.
- 5) n-Alkylcycloalkanes, with an odd number of carbon atoms in the side chain, are attacked initially by methyl hydroxylation and oxidation to form

the corresponding acid, which is then subjected to β -oxidation through cycloalkane carboxyl-CoA as an intermediate. 6) n-Alkylcycloalkanes, with an even number of carbon atoms in the side chain, are also degraded by oxidation to the corresponding fatty acid followed by β -oxidation. Molecules such as cyclohexylbutyric acid and cyclohexylacetic acid also enter this route, the β -oxidation phase of which is blocked with the formation of the (1'-hydroxyalk-1'yl)acetyl-CoA. Further metabolism of this intermediate is catalyzed by lyase, which cleaves the carbon-carbon bond linking the side chain to the ring with the formation of acetyl-CoA and the cycloalkanone.

A simplified pattern for biodegradation rates are highest for saturates, followed by the low molecular aromatics. High-molecular-weight aromatics and apolar compounds show extremely low rates [67]. The rates depend on both physiochemical and biological variables and many exceptions from these simplified patterns are described. For example, in an in situ continuous-flow system [68] or in a continuous-culture fermenter [69], degradation of all fractions of crude oil were at similar rates. Bioavailability limitations can result from poor uptake of nonhydrolyzable polymers or from the slow dissolution of highly apolar substances. Both of them are rate-limiting steps in bioremediation. Plastic (e.g., polyethylene or polystyrene), lignin, and humic compounds may be examples of nonhydrolyzable substances. Plastics can only be degraded if microorganisms are offered oligomeric plastics, which can passively diffuse into the cell [70]. Humics and lignin are nearly impossible to degrade by the most microorganisms and the average mean residence time of humus in aerobic soil range from 250 to 1,900 years [13–15]. However, the white rot fungi are able to degrade lignin and humus by extracellular oxidative enzymes [71]. For the dissolution of highly apolar substances the formation of emulsions and the release of biosurfactans through the microbial population are important [72]. Strong emulsifying activity was found in bacteria isolated from freshwater lakes [73] or waste water [74,75]. One more effector of the biodegradation rate is the temperature. Biodegradation rates are generally enhanced by increasing temperature to a maximum of 30°C to 40°C. Moreover the physical properties of the hydrocarbons are changing at different temperatures, for example, viscosity of toxic short alkanes is reduced at low temperature and their water solubility is increased. Nevertheless in cold [76–79] or hot [80] environments biodegradation is measurable (for review, see Margesin and Schinner [78]) Other effectors of the biodegradation rates are the concentration of oxygen and nutrients, the water activity, and the pH-value of the soil. The availability of oxygen in soils depends on different factors, such as, for example, oxygen consumption (microbial or chemical), water content of the soil, type of soil, and temperature. The water content of the soil not only effects the water availability for the microorganisms but also indirectly affects microbial activity due to limited solubility of oxygen in water and a rather slow diffusive transport of oxygen in water compared to air. Nutrients, (e.g., nitrogen or phosphorus) can be limiting in soil and increasing nutrients increased degradation of hydrocarbons in soil [81–84,79] (for review, see Harayama et al. [85]). Availability of water may limit biodegradation, Dibble and Bartha [83] reported optimal biodegradation at 30–90% of water saturation. Extreme pH-values are expected to have a negative influence and different studies found optimal degradation rates at neutral pH-values [83] (for review, see Margesin and Schinner [78]).

For full degradation of hydrocarbons in the environment a mixed population with broad enzymatic capacities is necessary. Leahy and Colwell [67] listed, based on the number of published reports, the most important hydrocarbon-degrading bacteria in soil environments: *Achromobacter* spp., *Acinetobacter* spp., *Alcaligenes* spp., *Arthrobacter* spp., *Bacillus* spp., *Flavobacterium* spp., *Nocardia* spp., *Pseudomonas* spp., and the coryneforms. Besides bacteria, fungi also are important organisms in the biodegradation of hydrocarbons. As the most common fungi in soil *Trichoderma* and *Mortierella* spp. and frequently *Aspergillus* and *Penicillium* spp. are listed. For agriculture soils Chaýneau et al. [86] found species of *Pseudomonas*, *Brevundimonas*, *Sphingomonas*, *Acinetobacter*, *Rhodococcus*, *Arthrobacter*, *Corynebacterium*, and species of the fungi *Aspergillus*, *Penicillium*, *Beauveria*, *Acremonium*, *Cladosporium*, *Fusarium*, and *Trichoderma*. The most active strains in the assimilation of saturates and aromatics were *Arthrobacter* sp., *Sphingomonas spiritivorum*,

Acinetobacter baumannii, *Beauveria alba*, and *Penicillium simplicissimum*. The part of the fungi on the total degradation is quantified in one study by Song et al. [87] as 13%. The part of algae and protozoa is unknown, but studies describe degradation of hydrocarbons by algae (e.g., Walker et al. [88], Cerniglia [89], Warshawsky [90], Pinto [91]; for review, see Semple et al. [92]) but seemingly not by protozoa. But it is known that protozoa can stimulate hydrocarbon degradation by grazing the bacteria [93–95].

Aerobic alkane metabolism is well studied in *Pseudomonas putida* Gp01 (formerly: *P. oleovorans*). A membrane-bound monooxygenase, a soluble rubredoxin, and rubredoxin reductase convert the alkane into an alcohol that is further oxidized into the aldehyde and then to the corresponding acid. The acid is fed into β -oxidation. Genes involved in the oxidation of the alkane are localized on the OCT plasmide [96,97]. Clustering and regulation of the genes in the operon varies among different bacteria. In *Acinetobacter* sp. strain M1, a dioxygenase, converts alkanes (C_{20} – C_{30}) and alkenes (C_{12} – C_{20}) directly to an aldehyde through a n-alkyl hydroperoxides without oxygen radicals. No rubredoxin and NAD(P)H are required [98,99]. In a *Rhodococcus* mutant the aliphatics are cis-desaturated, producing double bands mainly at the ninth carbon from the terminal methyl group [100].

Degradation of poly alicyclic hydrocarbons (PAH) with three or less rings as naphthalene, acenaphthene, pyrene, and chrysene are metabolized as only an energy or carbon source. PAH with 4 or more rings as benzo(a)pyrene are only metabolised cometabolic. PAH are relatively persistent because of the low solubility, the high sorption capacity, the production of toxic end-products or dead end-products, and the lack of cometabolic or inducer substrates (for review, see Juhasz and Naidu [101]). Naphthalene degradation in *Pseudomonas putida* strain G7 is encoded by three operons on a plasmide. The first operon encoded the enzymes for the conversion of naphthalene to salicylate by the introduction of molecular oxygen into the aromatic nucleus via naphthalene dioxygenase. Cis-naphthalene dihydrodiol, the product of the oxidation, is subsequently converted to salicylate. Salicylate is further degraded to acetaldehyde and pyruvate via catechol meta-cleavage by the enzymes of the second operon [102–104]. The third operon (nahR) encoded the regulator induced by salicylate [105]. Isofunctional gene sequences have been reported in other bacteria species like *Nocordia* [106], *Rhodococcus* [107], and *Mycobacteria* [108].

Under anoxic conditions mineralization of hydrocarbons is known since the late 1980s and many novel microorganisms utilizing hydrocarbons have been isolated. Anaerobic degradation of hydrocarbons was found with different electron acceptors. Microorganisms using nitrate, ferric iron, or sulfate as electron acceptors are known. Moreover, hydrocarbons are mineralized in syntrophic cocultures of various anaerobes or growth by anoxygenic photosynthesis (for review, see Spormann and Widdel [109]; Widdel and Rabus [110]). Because of the lacking oxygen for substrate activation, anaerobic microorganisms have involved other mechanisms. Anaerobic degradation of n-alkanes was shown under denitrifying [111], sulphate reducing [112–115], and methanogenic conditions with an enrichment culture [116]. Each strain showed a specialized capacity for a narrow range of n-alkanes that is utilized [109], as observed by aerobic n-alkane-degrading bacteria. The biochemistry pathway of the degradation of alkane seems different between the isolates. Activation of the alkane by a fumarate addition reaction is described for pure cultures of nitrate- and sulfate-reducing bacteria [117–119], and for sulfate enrichment culture [120]. In the denitrifying *Azoarcus*-like bacteria (Strain HxN1), the fumarate is added to n-hexane most likely by a radical mechanism forming a (1-methylpentyl)succinate [117,121]. For the following steps a pathway is proposed in which reactions are found analogous to those in the established conversion of succinyl-CoA via methylmalonyl-CoA to propionyl-CoA [121]. In contrast, the sulfate reducer strain Hxd3 degraded alkanes by transformation of the alkanes to a fatty acid through a mechanism that includes subterminal carboxylation at the C-3 position of the alkane and elimination of the two adjacent terminal carbon atoms [122]. Anaerobic methane oxidation, a pathway long-time proposed by strong geochemical evidence for net methane consumption in anoxic sediments, is based on methane

profiles, radiotracer experiments, and stable carbon isotope data. For more detail, see section 16.9 in this chapter.

16.3.3 AROMATIC COMPOUNDS AND LIGNIN DEGRADATION

Aromatic compounds are the second most abundant class of organic compounds in nature (next to carbohydrates). In plant material lignin makes the greatest fraction of aromatic compounds but also compounds with one or few aromatic nuclei occur with varying amounts (e.g., aromatic amino acids, several coenzymes, monomeric lignin precursors, flavonoids, phenyl glycosides, tannins, phytoalexins, and many other secondary metabolites). The great amount of aromatic compounds explains the fact that they are an important energy source for many microorganisms. In soils oxygen is available as a cosubstrate and electron acceptor, depending on the water content. In soil crumbs only the surface area is oxygenated and the center becomes anoxic due to both the oxygen respiration of the indigenous bacteria and the diffusion limitation of oxygen. Depending on the soil condition the aromatic compounds are degraded via completely different biochemical pathways.

In the presence of oxygen many soil bacteria (e.g., *Pseudomonas* sp.) degrade aromatic compounds with initial hydroxylation reactions catalyzed by mono- or dioxygenases. Long side chains on the aromatic nucleus are first dealkylated. These reactions funnel many aromatic compounds to few key intermediates like catechol and protocatechuate. Hydroxyl groups in ortho (catechol) or meta position (protocatechuate) of the aromatic nucleus favor ring cleavage reactions by dioxygenases. The products of the ring cleavage dicarboxylic acids are further degraded to acetyl-CoA and succinate.

Under anoxic conditions many of the oxygen-using soil bacteria degrade aromatic compounds by switching to different pathways elucidated during the last dozen years. Many novel enzymes and mechanisms have been discovered. The huge variety of aromatic substrates is again transformed into a few central intermediates that are amended to reduction of the aromatic ring. The first known central intermediate was phloroglucinol, which can be reduced with NADPH. Before ring reduction of phloroglucinol, unusual transhydroxylation reactions occur, which include 1,2,3,5 tetrahydroxylbenzene as cosubstrate funnelling pyrogallol and hydroxyhydroquinone into the phloroglucinol pathway [123]. Gallate, naturally the most abundant trihydroxylbenzene, is decarboxylated to pyrogallol [124].

The three isomers of dihydroxybenzene are degraded via different pathways. For resorcinol, already two different degradation pathways were described for fermenting bacteria and nitrate-reducing bacteria. A fermenting *Clostridium* strain directly reduced resorcinol to dihydroresorcinol, which is possible due to the two hydroxyl groups in meta position forming an unsaturated cyclohexenedione with an isolated double bond [125]. This double bond can be reduced to dihydroresorcinol, abolishing the aromatic character. A hydrolytic cleavage of the ring forms 5-oxocaproic acid, which is further degraded to acetate. Also the resorcinol carboxylates β - and γ -resorcyates are degraded by fermenting bacteria after decarboxylation to resorcinol [125]. The nitrate-reducing *Azoarcus anaerobicus* uses an entirely different biochemistry. Here an additional hydroxyl group is introduced to form hydroxyhydroquinone, which is further oxidized to hydroxybenzoquinone [126]. The further ring cleavage is not entirely clear yet. The latter pathway is also assumed for degradation of α -resorcyate which is very stable and cannot be easily decarboxylated [127].

Hydroquinone degradation studied with fermenting and sulfate-reducing bacteria showed a carboxylation to gentisate, which is activated to gentisyl-CoA [128,129]. In the fermenting bacterium *Syntrophus gentianae* gentisyl-CoA is dehydroxylated to benzoyl-CoA, which enters the benzoyl-CoA pathway (see below). Under sulfate-reducing conditions *Desulfococcus* did not reduce gentisyl-CoA to benzoyl-CoA, indicating possibly a direct reduction of the aromatic nucleus.

Anaerobic catechol degradation so far is only studied with a sulfate-reducing *Desulfobacterium* strain. First step is a carboxylation to protocatechuate, which is activated with CoA and subsequently dehydroxylated to benzoyl-CoA [130].

Degradation of phenol and monohydroxybenzoates is slightly different in fermenting bacteria, sulfate reducers, and nitrate reducers. The nitrate-reducing bacterium *Thauera aromatica* has been studied most intensively [131]. Phenol is first phosphorylated to phenylphosphate followed by a carboxylation to 4-hydroxybenzoate [132,133]. In fermenting and sulfate-reducing bacteria 4-hydroxybenzoate is an intermediate of phenol degradation too, although the primary phosphorylation remains unlikely due to the overall energy budget [134]. Further degradation is initiated by CoA activation, forming 4-hydroxybenzoyl-CoA, which is reductively dehydroxylated to benzoyl-CoA.

Degradation of 2-hydroxybenzoate appears in a similar manner to 4-hydroxybenzoate involving CoA activation and reductive dehydroxylation studied with a denitrifying bacterium [135]. For 2-hydroxybenzoate different degradation pathways were found in fermenting and nitrate-reducing bacteria. While the fermenters proceed similar to the above-described pathways for the other isomers of hydroxybenzoates (including CoA activation and reductive dehydroxylation [136,137]) oxidizes the denitrifier studied 3-hydroxybenzoate to gentisate (2,5-dihydroxybenzoate). Another hydroxylation and decarboxylation follows forming hydroxyhydroquinone, which appears to be a central intermediate in the transformation of phenolic compounds (resorcinol, α -resorcate, 3-hydroxybenzoate, gentisate and hydroquinone) (see above).

Aminated aromatic compounds show similar degradation steps to hydroxylated benzenes. Aniline (aminobenzene) was found to be carboxylated in para position to 4-amino benzoate [138]. The next steps are an activation with CoA and the reductive deamination to benzoyl CoA. The other isomers of aminobenzoate (2- and 3-aminobenzoate) were degraded in a similar matter [139].

Toluene degradation proceeds via a novel biochemical mechanism, including an addition of fumurate to the methyl group of toluene to yield benzoylsuccinate [140] (for review, see Heider et al. [141]). The benzoylsuccinate synthase generates a benzyl radical as intermediate to activate the inert molecule [142]. The further pathway proceeds via β -oxidation of benzoylsuccinate to benzoyl-CoA and succinate [143].

Benzoyl-CoA was found to be a central intermediate during degradation of many aromatic compounds (phenol, p-cresol, benzoate, hydroxybenzoates, toluene, and ethylbenzene) by many different anaerobic bacteria [143]. The reduction of benzoyl-CoA has to overcome the great stability caused by the resonance energy of the aromatic ring and the inertness of the C-H and C-C bonds in hydrocarbons. Most information about the benzoyl-CoA reductase is available for the enzyme of the denitrifying bacterium *Thauera aromatica*. It catalyzes the transfer of two electrons from reduced ferredoxine to benzoyl-CoA, yielding cyclohexa-1,5-diene-1-carboxyl-CoA [144,145]. Two ATP are stoichiometrically hydrolyzed to ADP for the two-electron transfer in *T. aromatica*. In fermenting and sulfate-reducing bacteria a less ATP-demanding mechanism is assumed due to the smaller overall energy yield.

The numerous strategies followed for the degradation of aromatic compounds by anaerobic bacteria appears to depend largely on the energy situation of the organism. In the case of the benzoyl-CoA pathway there are indications that fermenting bacteria and sulfate reducers use a variant of the pathway originally described for nitrate reducers that requires less energy for the benzoyl-CoA dearomatization [134].

Anaerobic benzene degradation has been reported for enrichment cultures [146] and recently two strains were isolated that grow with benzene as the sole carbon source and nitrate as electron acceptor [147]. The biochemistry of benzene degradation will be a future task. Similarly, for polycyclic aromatic hydrocarbon (PAH), mineralization under anoxic conditions has been observed under denitrifying, sulfate-reducing, and manganese-reducing conditions. For naphthalene also pure cultures have been isolated [148–150]. A carboxylation of naphthalene to 2-naphthoic acid was demonstrated as the first step of degradation [151,152].

Lignin is a highly irregularly linked polymer (mol mass $\geq 10,000$ dalton) with aliphatic and aromatic components. Lignin and its metabolites comprise many recalcitrant substructures (section 16.2 in this chapter) that retards degradation considerably. Lignin degradation is further aggravated by the fact that lignins recondense with degradation products to humic compounds that accumulate

and interact with their high cation exchange and sorptive binding capacity physicochemically with mineral soil components, especially clay. These interacting processes with the clay fraction especially retards lignin degradation.

Biodegradation of lignin and humic compound only occur in the presence of oxygen (section 16.3.3 in this chapter). In anoxic environments lignin, humics, peat, coal, and mineral oil have been conserved for millions of years (for review, see Field [18]).

In the 1990s anaerobic degradation of lignin and humics was postulated for soil-feeding termites. It was thought that the gut microorganisms of these termites are specially adapted for anaerobic degradation of humics as major soil compounds. Microelectrode studies of the termite gut system showed many oxic compartments in the termite gut, especially at the surface and in thinner compartments [153]. In the center of bigger compartments anoxic conditions are maintained by microbial oxygen respiration of the surrounding zones. These studies clearly revealed that oxygen is available in the termite gut for humics and aromatic compound degradation.

16.4 REDUCTION OF VARIOUS ELECTRON ACCEPTORS

Instead of oxygen, so-called alternative electron acceptors such as nitrate, manganese(IV), iron(III) in various oxides and hydroxides, sulfate, thiosulfate, sulfite, elemental sulfur, and carbondioxide are used by various metabolic groups of microorganisms. Also, in agricultural soils, depending on the water content and therefore the availability for oxygen, the anaerobic respiration processes are involved to a significant part in mineralization. In most agricultural systems the pool size of N is rather low and the plant uptake of mineral N is very efficient. Nevertheless denitrification is an important process in soils and mostly responsible for N_2O emissions from soil. Besides oxygen respiration, iron reduction is due to the high abundance of iron(III) oxides and hydroxides in soils, an important electron acceptor. Many iron-reducing bacteria have been isolated from soils and other environments and iron reduction was demonstrated to be of quantitative importance in anaerobic degradation of organic compounds. The biochemistry of metal reduction is under intensive study. Membrane-bound c-type cytochromes were found to be involved in iron reduction of *Geobacter sp.* [154–156] and *Shewanella sp.* [157,158]. In *Geobacter sulfurreducens* a periplasmic and extracellular c-type cytochrome was found to act as an electron carrier to ferric iron and also to partner bacteria [159]. Recently in *Geobacter sulfurreducens*, pilin-like filaments were found that function as nanowires to transfer electrons outside the cell onto insoluble electron acceptors, such as iron minerals [160]. The concentrations of Mn and S in soils are rather low and reduction of Mn(IV) sulfur, and SO_4^{2-} only play a minor role in mineralization of organic compounds. Depending on the availability of all the electron acceptors mentioned so far, the reduction of CO_2 by methanogenes or acetogens occurs. The sequential reduction of the electron acceptors can be explained by the thermodynamic theory [161–163] (for review, see Conrad [164]).

Some bacteria can reduce humic compounds as electron acceptors which can be reoxidized chemically with crystalline iron oxides. The model compound for humics anthraquinone-2,6-disulphonate (AHQDS) can be reduced by many bacteria, not only iron-reducing bacteria [165].

Due to increasing soil contaminations of agricultural soils with different metals from different sources like contamination through fertilizers [166,167] (for review, see McLaughlin et al. [168]), application of sewage sludge [169–171], mining activity, and air transport [172,173] detoxification by microorganisms were studied (for review, see Lovley and Coates [174]; Lovley and Phillips [175]). Many of the iron(III)-reducing bacteria are also able to reduce other metals, for example, soluble uranium U(VI) to less soluble U(IV) [176–179] (for review, see Lloyd and Macaskie [180]). Similarly, selenate (SeO_4^{2-}) can be reduced to selenite (SeO_3^{2-}) and elemental selenium (Se^0) [181–185] (for review, see Stolz and Oremland [186]), and the highly toxic and mobile Cr(VI) ions can be used as alternative e^- -acceptors by producing the less toxic and less mobile Cr(III) [187–190,178, for review, see Wang [191]; Cervantes et al. [192]). At least as important as the enzymatic Cr(VI)-reduction seems to be the abiological Cr(VI)-reduction through Fe(II) or Fe(0)

[193–195]. Soluble ionic mercury Hg(II) is microbially detoxified through reduction to volatile Hg(0) [196,197] (for review, see Hobman et al. [198]; Barkay and Wagner-Döbler [199]) whereby the aerobic Hg(II)-reduction pathway is evidently not linked to an energy-conserving electron transport. Technetium (TcO_4^-), released by nuclear power stations, is highly soluble and seems to be respired anaerobically by different bacteria like *Desulfovibrio* spp. [200,201], *Escherichia coli* [201,202], *Acidithiobacillus* spp. [203] and *Shewanella* spp. [204,205]. Heterotrophic bacteria and fungi isolated from a silty clay loam soil [206] and isolates like *Pseudomonas* spp. [207], *Shewanella oneideinis* [208], *Acidithiobacillus* spp. [209], and *Geobacter metallireducens* [210] turned the pale yellow color of a vanadium(V) into blue V(IV), and finally into black V(III).

Another class of electron acceptors for bacteria are represented by chlorinated and bromiated compounds. In the 1980s it seemed that hexachlorobenzene (HCB) could only poorly be degraded by enrichment cultures [211]. Meanwhile, many bacteria have been isolated that reductively eliminate the halogens of aliphatic (trichloroethane, tetrachloroethane, dichloroethane, chloroethane) and aromatic compounds (pentachlorophenol, isomers of trichlorophenol, dichlorophenol, and chlorophenol, and trichloro-hydroxyl-phenylacetate) in the presence of an electron donor like hydrogen or formate. The process is coupled to energy conservation by generation of a proton gradient during dehalorespiration. The membrane-anchored dehalogenase was isolated from various anaerobic organisms, for example, *Desulfotobacterium hafniense* [212], *D. dehalogenase*, *Dehalobacter restrictus* [213], *Dehalospirillum multivorans* [214], or *Dehalococcoides ethenogenes* [215], which all contain a corrinoid and Fe/S cofactor [216]. The dehalogenase from *Desulfomonile tiedjei* contains a heme and Fe/S cofactor for reductive dechlorination of 3-Cl-benzoate [217]. Recently the dechlorination of various isomers of tetra- and trichlorobenzene to tri- or dichlorobenzene was documented with a new isolate, which is phylogenetic related to *Dehalococcoides ethanogenes* [218]. This isolate used hydrogen as an electron donor for reductive dehalogenation and was not able to use nonchlorinated electron acceptors [218]. With dichlorophenol a deeply branching cluster within the Myxococcales, the facultative anaerobic bacterium *Anaeromyxobacter dehalogenans*, was isolated, which grew with dehalorespiration as well as with oxygen, nitrate, or fumarate as an electron acceptor [219].

For the reaction mechanism of the corrinoid-dependent reductive dehalogenase a one-electron transfer from Cob(I)alamine to tetrachloroethane followed by an elimination of a chloride anion, yielding a trichloroethenyl radical that combines with a H-radical to yield trichloroethane has been proposed [220].

16.5 AUTOTROPHIC METABOLIC DIVERSITY

Anabolism comprises all processes involved in the synthesis of the numerous cell compounds of microorganisms. In this respect the pentose phosphate cycle, the glycolytic pathway, the citrate cycle, the NH_4^+ -assimilation, the purin and pyrimidin biosynthesis, the fatty acid biosynthesis, and many other biosyntheses inclusively in precursor substances play a central role (for details, see e.g., Lengeler [221]; Madigan and Martinko [222]).

For autotrophic CO_2 -fixation various strategies have been evolved [221,223]. Similarly, for many autotrophic prokaryotes, green plants with active chloroplasts the Calvin cycle is of great importance for CO_2 -fixation. In phototrophic green nonsulfur bacteria and possibly in aerobic *Archaea* the 3-hydroxypropionate cycle is found [224], which is thought to be the evolutionarily oldest pathway for CO_2 -fixation. Other anaerobic *Bacteria* and *Archaea* fix CO_2 via the reductive citric acid cycle or the reductive acetyl-CoA pathway. The fixation of CO_2 (oxidation state +4) to cell carbon (oxidation state about 0) requires electrons, ATP, and NAD(P)H. Obligate autotroph CO_2 -fixing bacteria cannot utilize any other organic compound, while most facultative autotrophs shut down CO_2 -fixation when an appropriate organic substrate is available. An exception are Knallgas bacteria that utilize H_2 preferentially over organic substrate. Mixotrophs incorporate an organic carbon source and simultaneously fix CO_2 . Phototrophic facultative autotrophs preferentially

use organic substrates as a carbon source but fix CO_2 when reducing equivalents are available in excess. Oxygenic photoautotrophic cyanobacteria obtain energy and reduction power from the light-driven water cleavage and fix CO_2 with the Calvin cycle. Anoxygenic phototrophs living in the anoxic water column use light as an energy source and inorganic reduced compounds, for example, H_2S , Fe^{2+} or H_2 as electron donors. Chemolithoautotrophic aerobic bacteria dominate well-aerated niches depleted in organic compounds but enriched in NH_4^+ . Sulfide and iron(II) as electron donors for autotrophic metabolism is oxidized effectively by gradient organisms that compete with the chemical oxidation best at low oxygen concentrations. Oxidation of hydrogen, sulfide, iron(II), and ammonium is also catalyzed by anaerobic bacteria using various alternative electron acceptors.

16.6 PRODUCTION OF SECONDARY METABOLITES IN AGRICULTURAL SOIL

Respiration and fermentation are the key pathways in gaining energy in soils (primary metabolism; see the proceeding sections of this chapter). During energy conservation, the TCC intermediates form the basis for chemical differentiation leading to secondary metabolites. These are excreted into the environment e.g., able to induce antibiotic or growth-promoting reactions (fig 16.1). Involved in releasing plant growth-promoting metabolites (PGPM) are various free-living and symbiotic N_2 -fixing, siderophores-, hormones-, β -1,3-glucanases-, chitinases-excreting, and mineral phosphate or other nutrients-solubilizing microorganisms. Beneficial interactions with plants make plant growth-promoting bacteria (PGPB) promising candidates for further improvements [225] (chapter 6 in this volume).

Substances released by PGPB as auxines (indole acetic acids, IAA) implicate hormonal effects and contribute to a plant's endogenous pool. Together with the only by plants released, gibberellic acid and cytokinins are auxines involved in light and gravity orientated root and shoot elongation growth, in differentiation of vascular tissue, in apical dominance, in initiation of lateral and adventitious roots, and in stimulation of cell division. Ethylene, another plant and bacterial-produced hormone, formed during methionine metabolism, coordinates normal development in plants (growth of vegetative tissues such as roots, stems, and petioles). As small gaseous compounds effects C_2H_4 , additionally to flower senescence, leaf and petal abscission, and fruit ripening. It avoids further supernodulation of legumes and apparently improves the plant response to heavy metals, ozone, pathogens, and flooding stress.

Siderophores excreted by bacteria can function as plant growth-promoting compounds. By complexation siderophores solubilize iron(III). Ferric iron has relatively few competitors for the siderophore binding sites under natural circumstances, because most of the other metals are divalent, with aluminium as exception, and thus siderophores are very efficient in supporting iron nutrition. Plant siderophores (mugineic and avenic acid) differentiate as linear hydroxy- and amino-substituted iminocarboxylic acids from the bacterial ones [225].

Soil and rhizosphere microbes add to the plant growth-promoting compounds from which the environmental interactions behind are not fully understood. Often substances induce antibiotic reactions. From such secondary metabolites as antibiotics, pigments, pheromones, or toxins, it is believed that they are involved in mechanisms to improve survival in nature. Some of those secondary metabolites are enzyme inhibitors; others are immunomodulating agents, receptor antagonists, pesticides, or antitumor agents. The majority of those isolated so far (>7000 from cultures of microorganisms) are antibiotics, substances produced by a living organism that inhibit the growth of different other species of microorganisms at low concentrations. Antibiotic biosynthetic pathways may also be tied to amino acid synthesis or catabolism, nucleoside metabolism, or coenzyme synthesis.

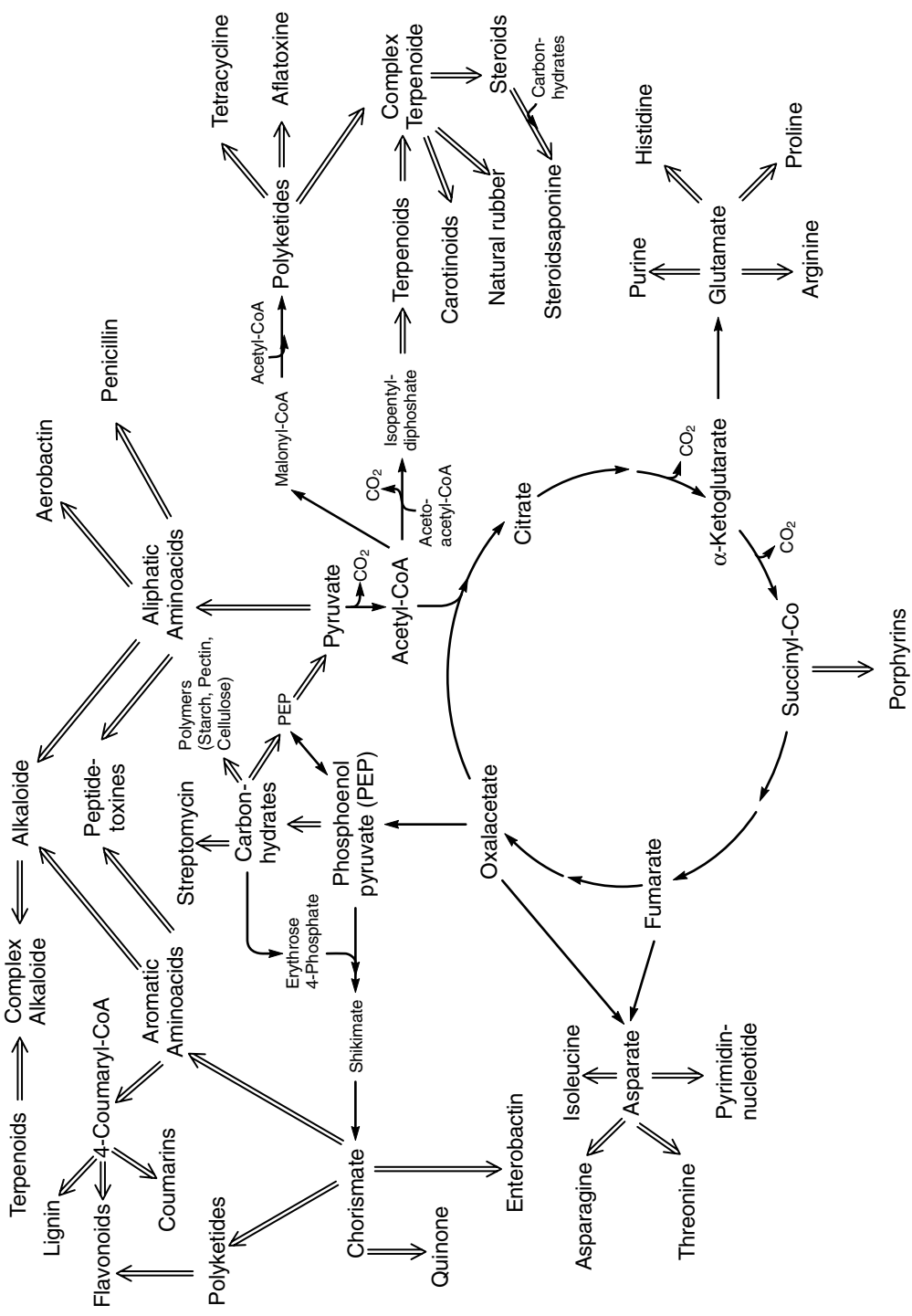


FIGURE 16.1 Biosynthesis pathways of secondary metabolites in microorganisms and plants.

Secondary metabolite production sets in at the edge of the exponential to the stationary growth phase and is mainly controlled by the available carbon, nitrogen, and phosphorus sources. Primary metabolites serve often as regulators and auto-inducers and play a role in quorum-sensing (chapter 18 in this volume). In feedback regulations they steer secondary metabolite production (e.g., in the case of *Streptomyces griseus* controls) an A-factor at least 10 proteins at the transcriptional level (e.g., the streptomycin 6-phosphotransferase). The plasmid-encoded secondary metabolite Agrocinn 84 of *Agrobacterium rhizogenes*, for example, reveals an antibiotic compound advantageous in bacterial–bacterial competition and improves the survival of *Agrobacterium rhizogenes* [226]. *Myxococcus xanthus*, which get nourishment in digesting other bacteria (e.g., *Escherichia coli* cells), produces antibiotics and high amounts of lytic enzymes. Because 60–80% of the myxobacterial isolates excrete antibiotics is this nutritional strategy seemingly widespread under these bacterial genus [226] (chapter 18 in this volume). With antibiotically active pigments (i.e., prodigiosin and violacein) *Serratia marcescens* and *Chromatium violaceum* strains protect themselves from being grazed by protozoa (chapter 11 in this volume). Microorganisms produce more than 150 compounds, called phytotoxins, which are active against plants or insecticides [226] (chapter 1 in this volume). All these examples exhibit secondary metabolite formation parallels as chemical differentiation morphological differentiation processes, for example, sporulation in bacilli and streptomycetes. Both forms of differentiation are frequently triggered by starvation and require chemosensors (chapter 18 in this volume).

16.7 METHODS TO ACCESS METABOLIC DIVERSITY

Climate (temperature, rainfall), soils (texture, grade of aeration), substrates (availability, degradability), and agriculture (fertilization, pesticide application, cultivation practices) can affect soil metabolic diversity. Such metabolic activity changes over time can be approached by estimating the humus contents [227], the soil microbial biomass [228], soil enzymatic activities (chapter 15 in this volume), or by quantifying the metabolite formation during the catabolic and anabolic soil processes (e.g., NH_4^+ , NO_3^- , N_2O , CO_2 , or CH_4) [19,229,230] (see preceding sections in this chapter; chapters 15 and 17 in this volume). Mostly used for assessing metabolic activity changes, the BIOLOG GN system in which the utilization of 95 different substrates is tested [231,232].

Recently developed methods employ phylogenetic markers to quantify specific gene activities in soils, for example, associated with nitrogen metabolism (for review, see Bothe et al. [233]), nitrogenase (*nifH*) [234,235], nitrous oxide reductase (*nosZ*) [235–237], and nitrite reductases genes (*nirK* and *nirS*) [237–240], (for review, see Philippot [241]). The degradation of specific organic compounds and the identification of resulting metabolites can be achieved in agricultural soils by labeling the original substrates with radioactive (e.g., ^{14}C) and nonradioactive isotopes (e.g., ^{13}C or ^{15}N). The decrease of the labelled original compound and the production of intermediates can be quantified by thin layer-, gas-, or liquid high-performance chromatography combined with scintillation counters or mass spectrometers [211,242].

Stable isotope probing allows the identification of microbial populations responsible for the metabolism of specific organic compounds. In stable isotope probing, either lipid biomarkers [243] or DNA [244] are extracted from communities incubated with ^{13}C -labeled compounds. If cells grow on the added compounds, their pool of macromolecules will be isotopically heavier compared to those of metabolically inactive organisms. This makes it possible to identify the organisms in one or two ways: (1) by mass spectrometry of labelled “signature” lipids [243]; or (2) by ultracentrifugation of community DNA, separation according to mass differences, followed by identification of rRNA genes in the isotopically heavier DNA pool by PCR, cloning, and sequencing [244].

Each of the listed techniques has distinct drawbacks and only the combination of the available methods allows a better insight into soil organismal and metabolic diversity. Comparing total organic carbon changes in agricultural production systems after organic fertilizer incorporation gives a rough idea if what might happen to the substrate [227] (chapter 20 in this volume). Changes in

microbial biomass formation estimated by the CHCl_3 -fumigation and total carbon extraction method [228] allow to quantify respiration coefficients. Thus microbial substrate consumption and activity changes of the soil microbial communities is determined by the above two methods. Based on growth and utilization of 95 carbon sources, the BIOLOG GN substrate utilization system, originally developed for the classification of bacterial isolates, has been adapted for characterizing the functional potential of microbial communities in soils. Further enhancement of the sole-carbon-source test (BIOLOG) was reached by measurement of carbon dioxide evolution in the microtiter plates, enabling the detection of an immediate response to a substrate (activity) rather than growth [245]. Yet, a comparison with molecular approaches (DGGE, TGGE [231]; chapter 5 in this volume) brought about the fact that substrate utilization profiles obtained with BIOLOG GN plates do not necessarily reflect the functional potential of the numerically dominant members of the microbial soil community used as inoculum. The limitations of carbon source utilization for assessment of functional diversity is critically reviewed by Preston-Mafham et al. [246]. Moreover, carbon substrate utilization profiles and fatty acid methyl ester patterns failed to confer a measurable soil microbial diversity change in a grassland site that has been enriched over 100 years with sewage sludge [247]. In plots treated 11 years with phenyl urea herbicides it was found that by combining the BIOLOG GN method with a 16S rRNA gene fingerprinting approach that phenyl urea decreased culturable heterotrophic bacteria and uncultured *Acidobacterium sp.* significantly [231]. The study yet could not clarify whether the herbicide caused the bacterial decline directly or by changing the macrofloral community that act intensively with soil bacteria (see the proceeding section this chapter; chapter 18 in this volume).

Stable isotope probing requires very high substrate concentrations and long incubation times, so the procedure actually resembles enrichment cultures [244]. First, in combination with microautoradiography and *in situ* hybridization (STAR- or MICROFISH) that allows more advanced molecular diversity studies in soils, stable isotope probing techniques might lead to exciting results in the future concerning our knowledge about agricultural impacts on soil metabolic diversity [248].

16.8 CONSEQUENCES OF LOST METABOLIC DIVERSITY

The metabolic diversity of agricultural production systems can be generalized in terms of organotroph, autotroph, phototroph, or lithotroph, reflecting the feature of electron donors are of importance. Usually, the utilization of complex organic compounds differentiates in microbial diversity, but lithotrophic organisms seem to contribute far more to microbial diversity than currently thought [249] (see preceding sections in this chapter). Recent theoretical and empirical analyses of the organismal diversity in soils indicate that soils harbor on the order of 7,000 different taxa at an abundance of approximately 10^9 cells per cubic centimeter [250] (chapter 5 and chapter 6 in this volume). A limiting factor may be a nutrient, a set of physical conditions such as temperature, pH, or salinity, or the lack of refuges from predation. Thus, the number in metabolic diversity in an environment is related to the number of niches. Looking at the partitioning of glucose into acetate and glycerol, it becomes obvious that metabolizing byproducts arising from the metabolism of glucose provide ecological opportunity for the evolutionary emergence of mutants with enhanced capacities to metabolize these byproducts. Over small spatial scales and in the absence of strict competitive hierarchies, diversity can be maintained through genotype–genotype interaction (chapter 7 in this volume). Diversity may get lost in shaken broth cultures, but also in cultivated and fertilized soils where the pore system and the distribution of nutrients are homogenized and the number of niches leveled [251,252] (chapter 1 in this volume). Agricultural measures as fertilization may initiate a change in soil metabolism. Atmospheric methane oxidation and nitrification were shown to be susceptible to ammonium [253,254] and agricultural cultivation [255] (chapter 17 in this volume). Decreasing yields and a changed atmospheric composition of greenhouse gases are observed (chapter 1 and chapter 17 in this volume). These *in situ* interactions in agricultural soils, especially in the soil rhizosphere environment, are better understood when the vividly developing spectrum of methods is applied in combination. A better insight into the rather complex interactions in the soil rhizosphere

environment can also be achieved by using a simpler model system, for example, the 6- to 7-mm-sized, less complex spermosphere, where *Fusarium* and *Pythium* species, the oomycetes *Achlya* and *Thraustotheca*, *Rhizoctonia solani*, *Penicillium*, *Trichoderma*, *Gliocladium*, *Cylindrocarpon*, *Cephalosporium*, *Cunninghamella*, *Mucor*, and *Helicocephalum*, fatty acid metabolizing bacteria, actinobacteria populations, and bacteria attracted by exudation as *Bacillus megaterium*, *Rhizobium* spp., *Burkholderia cepacia*, and *P. fluorescens* interact with each other in relatively short time frames and in rapidly changing developmental processes of plants and microbes [256].

16.9 POSTSCRIPT

Although the phylogenetic diversity of microorganisms is much greater than we know so far, the metabolic diversity of the so-far-undiscovered microorganisms is not likely to increase in a similar manner. Some exciting new metabolic pathways have been described in the last decade. Although anaerobic methane oxidation was postulated for a long time based on biogeochemical data [257,258] and theoretical considerations, the microorganisms involved in anaerobic methane oxidation have not been discovered before a few years ago. A close consortium of methanotrophic methanogenic archaea and sulfate reducers [259–261] was first discovered in deep sea methane sieves. The biochemical mechanism of methane oxidation and the interaction of the two microorganisms in the consortium is elucidated currently. Also recently described was the anaerobic ammonium oxidation (anammox) with nitrite as an electron acceptor in wastewater [262,263] and marine environments [264,265]. Since nitrite concentrations in environmental systems are very low, constant nitrite production by ammonium oxidation or nitrate reduction is postulated and in bioreactors clusters of anammox bacteria have been found next to clusters of aerobic ammonium oxidizers [266]. Both processes were found to be of high relevance in marine biogeochemical C and N cycling [267].

Lately described was the anaerobic corrosion process by surface-attached sulfate reducers able to directly utilize electrons from elemental iron oxidation to Fe(II) [268]. Another pathway fairly recently described is anaerobic iron oxidation by phototrophic [269] and nitrate-reducing bacteria [270,271].

However, a few new metabolic pathways that are feasible thermodynamically are likely to be discovered sooner or later. One of those is the anaerobic oxidation of ammonium, possibly with Mn(IV) as an electron acceptor.

The number of new synthesized organic compounds by the chemical and pharmaceutical industry will continuously increase. In addition, also more natural compounds from bacterial or plant synthesis will be discovered. Degradation pathways of all these compounds are likely to develop, for example, in combination and variation of the present pathways. To some extent degradation can be predicted from the structural similarity to known compounds. However, alternative ways of metabolism might evolve.

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17 Gaseous Emissions (CO_2 , CH_4 , N_2O , and NO) from Diverse Agricultural Production Systems

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17.1 INTRODUCTION

Agricultural soils are important sources and sinks of atmospheric carbon dioxide (CO₂), methane (CH₄), and major sources of oxides of nitrogen gases, nitrous oxide (N₂O) and NO_x (NO + NO₂), are present in the atmosphere in trace amounts [1–4]. Agricultural systems from high latitudes to the tropics contribute to global emissions of these gases, which are important to atmospheric chemistry and earth’s radiative balance because of the long atmospheric lifetimes of CH₄ and N₂O (~10 y for CH₄ and 120 yr for N₂O) and infrared absorption properties in the troposphere. Both species absorb terrestrial thermal radiation and thus contribute to greenhouse warming of the atmosphere. On a per-molecule basis and a 100-year time frame, CH₄ is about 23 and N₂O is about 296 times more effective at trapping infrared radiation than CO₂ [5]. CO₂ contributes approximately 40% of total radiative forcing and CH₄ and N₂O contribute ~20 and ~6 %, respectively [5]. Although nitric oxide (NO) does not directly contribute to the greenhouse effect by absorbing infrared radiation, it contributes to climate forcing through its role in photochemistry of hydroxyl radicals (OH⁻) and ozone (O₃) and plays a key role in air quality issues. In the past two centuries loss of CO₂ from soils due to land use change has contributed considerably to the increase in atmospheric CO₂ concentration [5]. Soil organic C loss trends in cropped soils can be reversed, however, by increased crop biomass production and reduced tillage [6].

17.1.1 CO₂

Net CO₂ flux to the atmosphere from terrestrial systems represents the balance between C inputs by autotrophic fixation and outputs by heterotrophic oxidation of organic material. On a global scale these processes are estimated to be in balance. A recent estimate of CO₂ flux to the atmosphere from soil respiration is 75 × 10¹⁵ g C yr⁻¹, while net CO₂ uptake by land plants is estimated to be the same [7]. However, at the smaller scales characterized by agricultural production units (i.e., tens to thousands of hectares) these processes may not balance. When CO₂ uptake by photosynthesis does not equal CO₂ loss through respiration, then the difference must reflect a change in C storage within the system, assuming no other inputs or losses occur. Historically, it has been well documented from several long-term studies that agricultural practices have shifted the balance between CO₂ fixation and respiration, resulting in a net loss of soil organic C, due to accelerated decomposition processes (see Paul et al. [8] for summaries and accompanying data). It is in this context that we present a discussion of soil CO₂ flux as a controlling process in soil organic matter change.

17.1.2 CH₄

The current global average atmospheric concentration of CH₄ is 1780 ppbv, more than double its preindustrial value of 800 ppbv [9]. The concentration of CH₄ in the Northern Hemisphere is about 100 ppbv more than in the Southern Hemisphere, indicating either greater sources or lower sink strength in the Northern Hemisphere [10]. The rate of increase in atmospheric CH₄ slowed from about 15 ppbv yr⁻¹ in the 1980s to near zero in 1999 [9]. The reason or reasons for this change is not known [11], but changes in organic matter input and field drainage may have contributed to the decrease [12].

About 70% of CH₄ production arises from anthropogenic sources and about 30% from natural sources. Biological generation in anaerobic environments (natural and human-made wetlands, enteric fermentation, and anaerobic waste processing) is the major source of CH₄, although losses associated with coal and natural gas industries are also significant. The primary sink for CH₄ is

reaction with hydroxyl (OH⁻) radicals in the troposphere [13,14]. Aerobic soils oxidize 10–20% of net CH₄ production, while a stratospheric sink of about 10% of production is suggested [15]. CH₄ emissions from tropical agricultural soils constitute a significant portion of annual global CH₄ emissions. Rice paddies, termites, biomass burning, and enteric fermentation are the main contributors [16]. In our discussions here we focus only on the soil sources and sinks associated with agricultural production of food, forage, and fiber.

17.1.3 N₂O

The current atmospheric N₂O concentration, ~318 ppbv, has increased from ~275 ppbv in 1900. Most of this increase has occurred during the past 50 years with a near linear increase of ~0.7 ppbv yr⁻¹ [17]. An increase of 0.2–0.3% in atmospheric concentrations would contribute about 5% to greenhouse warming [18]. Nitrous oxide is also involved in the depletion of the ozone layer in the stratosphere, which protects the biosphere from the harmful effects of solar ultraviolet radiation [19]. Doubling the concentration of N₂O in the atmosphere would result in an estimated 10% decrease in the ozone layer and this would increase the ultraviolet radiation reaching the earth by 20% [20]. This could result in an increased risk of skin cancer and other health problems [21]. Given the relatively long atmospheric lifetime for N₂O [22–24], there are justifiable reasons for concern.

The concentration of N₂O in the atmosphere is increasing, as a result of biotic and anthropogenic activities, at the rate of about 4 Tg N₂O-N yr⁻¹ (1 Tg = 10¹²g) above the rate of decomposition in the stratosphere [16]. Kroeze et al. [25] estimated that total global N₂O emissions in 1994 were approximately 17.6 Tg N, with ~55% (9.6 Tg) of the total arising from relatively natural terrestrial and aquatic systems and ~8 Tg derived from anthropogenic sources. Of the human-initiated N₂O emissions, ~70% is thought to result from emissions from agriculture, both crop and livestock production [25].

Data drawn from temperate agricultural crop production systems demonstrate that N₂O is emitted in response to N fertilization. Nitrogen fertilizer use and biological N-fixation are projected to continue to increase during the next 100 years [26] to support global food production needed by a rapidly expanding population [27]. Worldwide synthetic fertilizer consumption was ~85 Tg N in 1999, with ~56 Tg N consumed in developing countries [28]. Global N fertilizer consumption is projected to reach 105 Tg N by 2030 [29]. Thus, the current linear trend of increasing atmospheric N₂O concentrations is likely to continue over the next several decades.

17.1.4 NO_x

The release of nitrogen oxides (NO_x = NO + NO₂) has accelerated during the last few decades primarily through the increase in fossil fuel combustion [30,31] and to a lesser extent through tropical forest clearing [32]. NO is a short-lived species in the troposphere (hours to days) and thus has mostly local or regional effects on photochemistry. NO is a strong oxidant and contributes indirectly to climate, forcing through a series of concentration-dependent cycles with other atmospheric oxidants, ozone (O₃), and hydroxyl radicals (OH⁻) during the oxidation of carbon monoxide (CO), CH₄, and nonmethane hydrocarbons. Increasing NO emissions are contributing substantially to observed increases in O₃ concentrations in the Northern Hemisphere [33,34]. Detailed descriptions of the complex interactive cycles of NO in the atmosphere can be found in many references [31,35,36,37].

Several authors (e.g., Williams et al. [37]; Davidson and Kinglerlee [38]) have used an inventory approach to estimate that about 20–40% of global NO emitted to the atmosphere is derived from soils. Estimates of annual soil-derived NO emissions range from 5.5 [34,39] to 21 Tg N [37,38]. Yienger and Levy [39] used a combination of inventory and modeling to estimate global NO emissions from soils to be 10.2 Tg yr⁻¹. Potter et al. [40] also used a modeling approach and

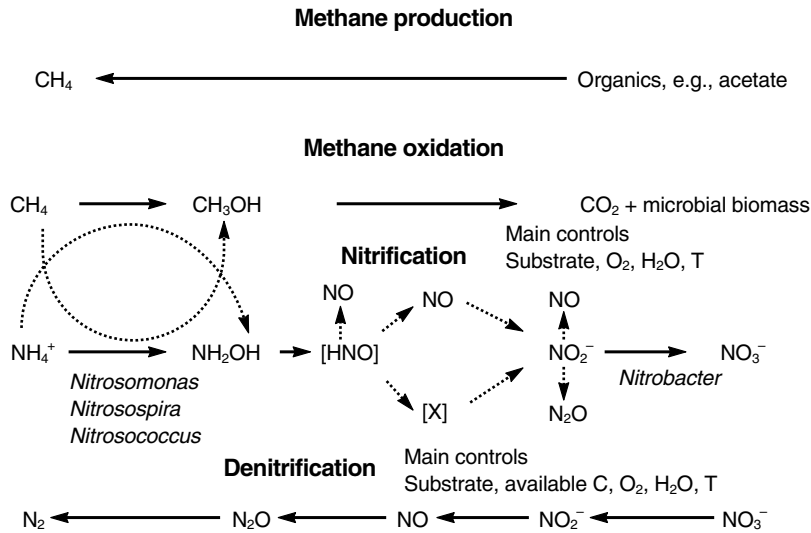


FIGURE 17.1 Diagram of interrelationship of processes responsible for CH_4 production and oxidation, and nitrification and denitrification (adapted from Knowles [95]; Firestone and Davidson [105]; Venterea and Rolston [118]; Mosier et al. [151]).

estimated the global flux as 10 Tg yr^{-1} . As Davidson and Kingerlee [37] pointed out, the estimate of Yienger and Levy [39] is probably a bit low for some biomes, particularly tropical savannas. Therefore it is probably best to think of this estimate as a reasonable lower bound.

Estimates of soil fluxes do not represent biosphere fluxes to the atmosphere. NO rapidly oxidizes to NO_2 , which adsorbs readily onto surfaces in the plant canopy. Taking this adsorption into account the estimate of Yienger and Levy [39] decreased to 5.45 Tg yr^{-1} of NO_x ($\text{NO} + \text{NO}_2$) emitted to the atmosphere from ecosystems. Applying this same adsorption coefficient to the Davidson and Kingerlee [38] estimate would lower their estimate to 13 Tg yr^{-1} . Thus the best estimate available right now would suggest that soil emissions are between 10 and 20 Tg yr^{-1} and that ecosystem emissions to the free atmosphere are at most 40% lower than the soil emissions.

While the magnitude of the soil flux may be difficult to pin down with any precision at the moment, the likely future trend of emissions is less difficult to predict. The microbial processes responsible for NO emissions in soils are the same processes responsible for N_2O emissions, namely nitrification and denitrification (fig. 17.1). NO emissions are related to N turnover rates and increases in N cycling in soils have contributed to the increases in NO emissions during the past century. We fully expect this trend to continue. Other factors have increased N cycling rates such as conversions of forests and grasslands to croplands. Changes in global climate may affect temperature and moisture, which will directly influence N cycling. Increased N input into the soil system via atmospheric deposition will also increase N cycling. All of these changes are likely to increase soil NO emissions in the coming decades.

17.1.5 CHAPTER OBJECTIVES

Presented in this chapter is a brief overview of the processes involved in the production and consumption of CO_2 , CH_4 , N_2O , and NO in the soil. A general discussion of soil CO_2 relationships is presented and data are provided from a number of selected field studies to compare and contrast CH_4 , N_2O , and NO exchange in diverse climates and crop production systems around the world.

17.2 PRODUCTION AND CONSUMPTION OF CO₂, CH₄, N₂O, AND NO

The soil microbial processes responsible for the production and consumption of CO₂ and CH₄ and production of N-oxides are the same in all parts of the globe, regardless of climate. Different species may predominate in specific situations and organisms adapted to a specific climate may respond differently when exposed to changing environmental conditions. Because of the ubiquitous nature of the basic enzymatic processes, we can discuss soil respiration, nitrification/denitrification, and methanogenesis/methanotrophy in general and not be confined to discussing the process for specific climatic conditions (fig. 17.1). Soil organisms responsible for trace gas consumption and production that are adapted to tropical climates typically respond near linearly to temperature between 20 and 40°C [41]. Discussion of flux rates or trace gas exchange in this chapter will follow the convention that fluxes to the atmosphere are positive while fluxes from the atmosphere to the land surface are negative.

17.2.1 PRODUCTION AND CONSUMPTION OF CO₂

Soil processes are typically a net source of carbon dioxide. Although CO₂ consumptive processes such as autotrophic fixation by algae, lichens, or chemolithotrophic bacteria occur, the magnitude of CO₂ fixed by these processes is generally small. Weathering of silicate minerals and formation of carbonates also consume atmospheric CO₂, as has been observed in temperate grasslands and desert scrubland [42]; however, the magnitude of this process is also small relative to soil CO₂ production.

Carbon dioxide flux from the soil is largely due to biological processes, although inorganic processes such as dissolution of carbonates due to soil acidification can also serve as a source of atmospheric CO₂. This latter process may be significant in soils requiring lime additions to maintain pH [43,44]. Typically, CO₂ flux from soil is recognized as the result of two processes: root respiration, and decomposition of organic materials by soil micro and macro fauna. Root respiration results in the release of CO₂ from the metabolism of plant root cells; however, because of methodological problems, it is difficult to determine root cell-derived CO₂ from the CO₂ derived from decomposition of root exudates in the rhizosphere. Thus, these two processes are often not distinguished in discussions of soil respiration [45].

17.2.1.1 Root Contributions to Soil CO₂ Flux

The contribution of root respiration to total soil respiration is dependant upon vegetation type, growing patterns, season, soil, climate, and management conditions. In a review of 50 studies of a variety of ecosystems, Hanson et al. [46] observed that, for forested systems, the mean contribution of root respiration to total soil respiration ranged from 45–56% depending on how measurements were performed and how the data were integrated. In agricultural systems, annual contributions of root respiration to soil CO₂ flux are lower. Rochette et al. [47] report that for maize in Eastern Canada root respiration was zero over the first 30 days from planting, but during the next 30 days of plant growth the contribution of root respiration increased linearly to a maximum of 45%, where it remained constant until plant senescence. Total soil CO₂ flux during the 160-day period from planting to harvest was 5.5 Mg CO₂-C ha⁻¹, with root respiration accounting for 28.7% of this total seasonal soil respiration. Thus in agricultural systems, where annual crops are planted, yearly contributions of root respiration to total soil respiration appears lower than in systems with perennial vegetation.

17.2.1.2 Organic Matter Decomposition Contribution to Soil CO₂ Flux

Decomposition of organic matter is carried out by the soil biota, and predominately by soil microorganisms. The direct or proximal factors controlling soil microbial activity are shown in

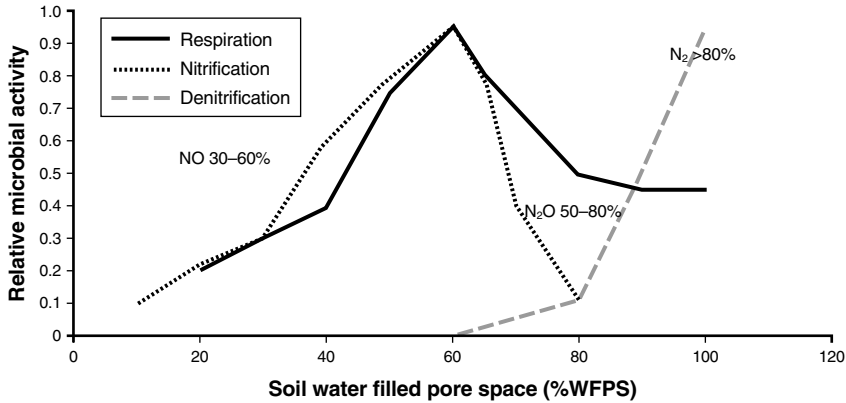


FIGURE 17.2 Effect of soil water-filled pore space on soil microbial processes and NO and N₂O emissions (adapted from Linn and Doran [67]).

figure 17.2, and include temperature, water, oxygen content, substrate availability, and substrate quality. The effects of these factors are influenced by distal factors such as location in the landscape, vegetation type, soil texture, and management. Expression of microbial activity at any given scale is influenced by the interactions of these factors. The simplistic representation in figure 17.2 does not show the multitudes of interactions between and among the proximal and distal factors, and a detailed discussion of these interactions is beyond the scope of this presentation; however, a general overview of these controls is provided below.

17.2.1.3 Temperature

Seasonal changes in CO₂ flux have been reported to follow seasonal temperature trends [48–52]. On a shorter time scale, diurnal changes in soil CO₂ flux have generally been observed to follow soil temperature [53,54], with maximum CO₂ fluxes occurring in the mid-afternoon, and minimum fluxes occurring in the early morning. Davidson et al. [55] reported a significant diurnal relationship at an active pasture site, but observed no significant temperature/CO₂ flux relationships at a primary forest or a degraded pasture site. Similarly, Jensen et al. [56] observed that diurnal CO₂ flux was not significantly correlated to 5 cm soil temperature.

Laboratory studies have generally shown an exponential relationship between temperature and CO₂ production. These relationships are often described using a Q₁₀ relationship, where the Q₁₀ factor is the ratio of respiration rates observed at temperatures differing by 10°C. Fang and Moncrieff [57] evaluated a number of mathematical relationships using a Q₁₀ criterion, and observed that model-derived Q₁₀ factors ranged from 1.5 to 8.8. Typically, temperature/respiration relationships observed in the laboratory have been determined under conditions of constant temperature across time and soil depths, and with homogenized soil without surface residues. However, in the field, where temperatures vary with time and soil depth, and where surface residues may be present, CO₂ flux/temperature relationships may be more complex.

A substantial body of literature exists on Q₁₀ factors that can be applied to soil CO₂ flux, but little information exists concerning where the temperature should be measured in the field. Often the temperature at the 0.05 or 0.10 m soil depth has been used, presumably with the assumption that the biological activity at these depths is representative of the average and that CO₂ flux will be a reflection of activity at these depths. Unlike laboratory settings, where temperature is typically held constant and soils are often homogenized, in the field biological activity and soil temperature vary in both time and space. Thus, application of a given temperature correction algorithm will yield different results depending on where temperature is measured relative to where the biological

activity is occurring. The lack of coincidence between the depth of measured soil temperature and activity could explain the poor correlations occasionally observed in the field [58]. Paul et al. [59] reported better correlations of CO₂ flux with air temperature than with modeled soil temperatures and recently, Parkin and Kaspar [60] observed that diurnal 5 cm soil temperature and CO₂ flux were out of phase. Their results indicated that soil CO₂ flux had a stronger temporal coincidence with air temperature than with soil temperature, suggesting that CO₂ flux patterns at their no-till site was dominated by near-surface processes. Soil temperature regime will be influenced by position in the landscape relative to solar incidence, texture of soil that controls the absorption and reflectance of impinging radiation, shading by vegetative cover, and management practices such as tillage and residue placement.

17.2.1.4 Water

Soil water availability exerts a direct control on microbial activity and growth through matrix and osmotic stresses that affect intercellular water potential and solute uptake. Microbial tolerance to water stress varies greatly among microorganisms, with maximum water potentials ranging from -1.5 MPa to -20.0 MPa for bacteria and up to -65.0 MPa for some fungi [61]. In addition to its effect on microbial osmoregulation, soil water indirectly affects microbial activity by regulating solute and gas transport [62]. Solute availability to microorganisms is impacted by both diffusion and mass transport processes. Limitations on microbial activity related to solute transport and availability are manifested at much smaller changes in water potential than the direct effects on osmoregulation.

Another important effect of water on microbial activity is related to the temporal dynamics of water. Wetting and drying cycles can result in burst of CO₂ from soil [63]. Rapid increases in soil water content can result in lysis of microbial cells due to osmotic shock [64]. The cytoplasmic components released can undergo rapid decomposition by surviving organisms resulting in a burst of soil respiration [65].

Water potential effects, gas exchange, and particularly oxygen availability exert a major control on microbial decomposition of soil organic matter. Decomposition of organic matter in soil generally proceeds faster under aerobic conditions than in anaerobic conditions. Thus, as soil water content increases, oxygen transport in soil decreases. In this regard, soil texture and position in the landscape have important influences on soil water content. This effect is evident by observations of higher carbon levels in poorly drained soils [66].

A derived variable that has been used to integrate the relationships between soil water content and soil aeration state is water filled pore space (WFPS). Water-filled pore space is a quantitative measure of the fraction of soil pores that are filled with water. The relationship between WFPS and respiration was delineated by Linn and Doran [67], who observed that maximum respiration occurred at approximately 60% WFPS (fig. 17.2). This relationship was relatively invariant over a range of soil types investigated.

17.2.1.5 Substrate

Availability of substrate is also a prime controller of soil microbial respiration. It is generally observed that organic C degradation proceeds according to first-order kinetics, where degradation rate is proportional to the amount of substrate available. Although substrate degradation and CO₂ production may not be directly comparable since part of the substrate consumed is used to create microbial biomass, the efficiency of biomass production (measured a fraction of biomass formed per unit substrate degraded) typically only varies in a narrow range (0.4 to 0.6). Thus, as C inputs to the system increase, either through crop residues and root biomass or by exogenous additions of organic materials, CO₂ production also increases. This response does not necessarily mean that all added C is converted to CO₂. In long-term experiments it has been generally observed that as

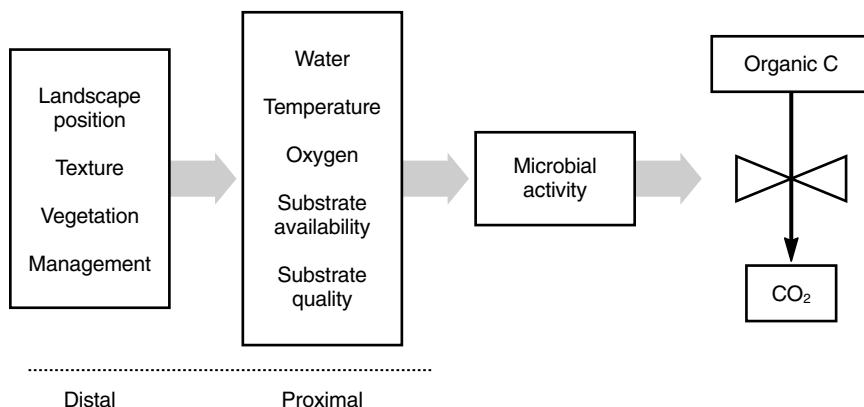


FIGURE 17.3 Direct (proximal) and indirect (distal) factors controlling microbial activity in soil.

C inputs soil increase, soil organic matter increases [68,69]. An exception to this generalized response appears to be in soils with high initial organic C content [70].

Substrate availability is not only a function of C inputs, but is also related to accessibility. Carbon accessibility in soil is influenced by three mechanisms: 1) physical protection, 2) chemical stabilization, and 3) biochemical resistance [71]. Physically protected carbon is material trapped inside of soil aggregates that is not accessible to microbial action [72–75]. Chemically stabilized C is in the form of organic matter bound to soil, especially clays [71,76]. Biochemical availability of organic matter relates to the susceptibility of the organic material to enzymatic attack. Plant residues are composed of soluble components (amino acids, sugars, amino sugars), protein, hemicellulose, cellulose lignin, and protein. Each of these components differ with regard to their decomposability, with soluble components being the most rapidly degraded, followed by hemicellulose, cellulose, and finally lignin. Thus, the composition of the plant residues returned to soil will influence the decomposition rate [77,78].

The proximal factors that exert direct controls on soil microbial activity are in turn impacted by the distal factors shown in figure 17.3. The distal factors are susceptible to varying degrees of anthropogenic control. Agricultural management offers the greatest opportunities for indirect control of microbial activity. Changes in agricultural management practices such as clearing, drainage, tillage, crop selection, grazing, crop residue placement, organic amendments, fertilization, and flooding can modify both organic matter inputs and decomposition, and thereby impact CO₂ flux from soil. A detailed discussion of management effects on soil C change and CO₂ flux is beyond the scope of this chapter, but several recent volumes have been devoted to this topic (e.g., Paul et al. [8]; Bowman [79]). An excellent overview of management influences on soil C is presented by Paustian et al. [80], while Mosier [81] discusses the global implications of soil management (deforestation and cultivation).

17.2.2 CH₄

Atmospheric CH₄ is produced by a wide variety of natural and anthropogenic processes in which agriculture plays a major part. CH₄ emissions from tropical agriculture, namely from rice paddies, termites, biomass burning, and enteric fermentation, constitute a significant portion of annual global CH₄ emissions. Upland agricultural soils are a CH₄ sink. The net flux (emission or consumption) of CH₄ will vary depending on the nature of the agricultural system and the management practice adopted in the system. Measurements made at various locations of the world show that there are large temporal variations of CH₄ flux, which differ with soil type, application of organic matter, and mineral fertilizer.

17.2.2.1 CH₄ Production in Soils

Methane production occurs only under highly anaerobic conditions such as typically occur in natural wetlands and lowland rice fields. There are many distinguishing factors that differentiate wetlands from upland, aerobic soil systems. The main processes that occur in flooded soils can be regarded as a series of successive reduction and oxidation (redox) reactions mediated by different types of microorganisms. Flooding alters the character of the microbial flora in soils by decreasing O₂ concentration. Fermentation is one of the major biochemical processes responsible for organic matter degradation in flooded soils. The main products of the fermentation process in flooded soil are ethanol, acetate, lactate, propionate, butyrate, H₂, N₂, CH₄, and CO₂. The latter three gases usually constitute the largest portion of the gas phase of flooded soils.

The major pathways of CH₄ production in flooded soil are the reduction of CO₂ with H₂, with fatty acids or alcohols as the hydrogen donor, and the transmethylation of acetic acid or methyl alcohol by methane-producing bacteria [82,83]. In paddy fields, the kinetics of reduction processes are strongly affected by the composition and texture of soil and its content of inorganic electron acceptors. The period between flooding soil and the onset of methanogenesis can vary with different soils. However, it is unclear if soil type also affects the rates of methanogenesis and CH₄ emission when steady-state conditions have been reached [83].

The redox potential (E_h) directly controls the production of CH₄ in soils, with CH₄ typically being produced at E_h below -100 mv [84]. The E_h of the soil gradually decreases after flooding. Takai et al. [85] and Yamane and Sato [86] showed that CH₄ was not emitted from flooded paddy soils until the E_h fell below ~200 mv. There is a correlation between the soil redox potential and CH₄ emission [87–89]. Typically application of rice straw to paddy fields significantly increases CH₄ emission compared with an application of compost prepared from rice straw or chemical fertilizer [89].

17.2.2.2 CH₄ Transport

There are three mechanisms for the transfer of CH₄ from rice paddy soil to the atmosphere. Methane may be lost through ebullition (in bubbles from paddy soils); it may diffuse across the water surface; or it may be transported through the rice plant aerenchyma. Release to the atmosphere through the shoot nodes, which are not subject to stomatal control, is generally the most important emission mechanism, accounting for more than 90% of the total CH₄ emission from rice paddies [88,90–92]. During the course of the rice-growing season, 20–90% of the CH₄ produced in the flooded soil is oxidized before being released to the atmosphere [12,93]. Although CH₄ flux rates are a function of the total amount of CH₄ in the soil, there is the possibility that the gas may be consumed in the thin oxidized layer close to the soil surface and in deep floodwater. Methanotrophic bacteria can grow with CH₄ as their sole energy source, and other soil bacteria (e.g., *Nitrosomonas* species) consume CH₄ [94]. As a small amount of CH₄ is dissolved in water, it may be leached to ground water.

17.2.2.3 CH₄ Oxidation

Knowles [95] describes in detail microbial pathways of CH₄ oxidation in soil methanotrophic organisms. He notes that all methanotrophs isolated thus far are obligate aerobes. This seems reasonable since the enzyme responsible for the initial step in CH₄ oxidation is a monooxygenase enzyme (MMO) that requires molecular O₂. Knowles [95] also describes the biochemical pathway for CH₄ oxidation and ammonium oxidation. The affinity of a methanotroph for CH₄ governs its ability to compete for CH₄ at low concentrations. Bender and Conrad [96] suggest the existence of two different types of methanotrophic bacteria in soil. One functions at high CH₄ mixing ratios and has a relatively low affinity for CH₄ and the other population, which exhibits low thresholds and high affinity for CH₄, oxidizes atmospheric CH₄ at mixing ratios at or below atmospheric concentrations. These organisms can actively oxidize CH₄ at concentrations as low as 0.1 ppmv.

Most methanotrophs are mesophiles, but some, such as *M. capsulatus*, are tolerant of high temperatures up to 50°C [95]. The temperature quotients (Q_{10}) for consumption were in the range of 1.4 to 2.1, in contrast to much higher values for CH_4 production. The low activation energy required by methanotrophs buffers temperature effects and explains the relatively high rates of CH_4 oxidation observed in cold soils [97,98].

Most organisms that have MMO appear to have the ability to co-oxidize ammonia and to contribute to nitrification. The MMO of methanotrophs and the ammonia monooxygenase (AMO) of nitrifiers have similar substrate specificities, and apparently CH_4 and NH_3 are competitive substrates for both enzymes [95]. The autotrophic nitrifiers and methanotrophs occur in similar soil habitats and may compete for O_2 , CH_4 , and NH_3 . Knowles [95] notes that methanotrophs can also participate in gaseous N oxide metabolism. Methane uptake is controlled by the interplay of biotic and abiotic factors. The major factors controlling total consumption rate are potential biological demand and diffusion. Thus, prediction and explanation of the CH_4 consumption rate hinges on understanding these two processes. Diffusion rate is regulated by physical factors, while biological demand is regulated by both the physical and chemical environments. Under any particular situation, either biotic or abiotic factors may provide the proximate limitation on CH_4 uptake rate.

Using a database that included measurement of the soil-atmosphere exchange of CH_4 from a variety of studies in temperate and tropical native and managed ecosystems, Del Grosso et al. [99] observed that oxidation of CH_4 in the soil is clearly dependent upon soil water-filled pore space (WFPS). The optimum soil WFPS was dependent upon soil texture with the optima of about 7.5% for coarse textured soils and 13% for fine textured soils. It is clear that CH_4 oxidation is controlled by gas diffusivity and biological activity. When soil water content is greater than the optimum, gas diffusivity limits CH_4 oxidation, but when soil water content is below the optimum, water stress limits biological activity [99].

17.2.3 N_2O AND NO

Nitrous oxide is produced in “natural” and agricultural soils almost exclusively as a result of microbial processes in the soil. As ammonium is the initial mineral N product formed during organic matter mineralization and most fertilizer used worldwide is ammonium based (e.g., urea, ammonium sulfate [28]), the suite of microbiological reactions that result in the release of gaseous N products need to be considered. The main microbial reactions involved in the production of N_2O are nitrification and denitrification and we will discuss them separately in the pages that follow. We hope that the reader will keep in mind that although nitrification is basically an aerobic process and denitrification is essentially an anaerobic process that both processes can take place in the soil in close proximity under soil conditions that are amenable for upland crop and forage production.

When atmospheric scientists first expressed concern that nitrous oxide emissions into the atmosphere as a result of fertilizer use would lead to destruction of the ozone layer, [20,100], it was thought that nitrous oxide was produced mainly from the denitrification, under anaerobic conditions, of nitrate produced [101]. However, research in 1978 showed that significant nitrous oxide was emitted from aerobic soils during the oxidation of ammonia to nitrate (called nitrification; see figure 17.1 [102,103], and subsequent work has shown that nitrification is a major source of nitrous oxide in upland soils [104].

It is accepted that an increase in the amount of nitrogen being cycled will result in an increase in the emission of nitrous oxide. The production of nitrous oxide and nitrogen gases by these two processes can be compared with gases leaking from a pipe [105]. The greater the flow through the pipe (the nitrogen being cycled), the greater is the fraction leaking through the holes (the emission of N_2O).

17.2.3.1 Process-Level Controls on Gaseous Emissions of N

Emissions of NO and N₂O from soil have been conceptualized as leaks of N flowing through metaphorical pipes of nitrification and denitrification [105]. Leaks in the pipe are significant only when there is ample N flowing through the pipe, as occurs in naturally fertile soils or soils that have received significant N inputs from atmospheric deposition or from fertilizers. Hence, emissions of either gas tend to be low where inputs of N or rates of N mineralization are low relative to plant demand [106]. The relative sizes of the holes in the pipes illustrate the importance of environmental factors such as soil water content, acidity, and ratios of electron donors to electron acceptors [105]. Soil water content is a particularly strong controller of the sizes of the “holes in the pipe” by regulating the redox conditions of the soil [58,106]. Under well-drained, aerobic soil conditions, NO is the dominant gas, usually from nitrification. Under wetter conditions, where anaerobic microsites become increasingly abundant, N₂O from nitrification and denitrification becomes the dominant gas. Under very wet conditions with little oxygen diffusion, N₂ from denitrification is the dominant end-product. This conceptual model is simplistic in that it ignores heterotrophic nitrification and details of whether autotrophic nitrifiers produce these gases from oxidative or reductive pathways [105]. Nevertheless, this model captures the two most important regulating factors that distinguish conditions for large and small fluxes of NO and N₂O. First, reducing overall emissions requires that N availability be managed so that it seldom exceeds plant demand (e.g., less N entering the nitrification and denitrification “pipes”). Avoiding emissions trading (e.g., decreasing N₂O emissions by reducing excess water, but thereby increasing NO emissions) requires understanding of the dominant factors affecting the ratios of the gaseous products (the sizes of the holes in the pipes).

The depth of production and the gaseous diffusivity of the soil between the site of production and the soil surface are important for all three of these gases. When diffusivity is low and/or the diffusion path is relatively long, the probability of efflux is lower and the probability increases that NH₃/NH₄⁺/NO₃⁻ immobilization can first occur or that NO and N₂O can be reduced to N₂. This conceptual understanding can be applied to the placement of N fertilizers, thus explaining why emissions are often higher following surface applications and lower when fertilizer-N is worked into deeper soil layers by various means [107].

17.2.3.2 Nitrification

Nitrification is the oxidation of ammonium to nitrate (fig. 17.1). Most commonly nitrification is a chemolithotrophic process that consists of the conversion of ammonia to nitrite, which is then converted to nitrate by a second group of bacteria. The ammonia-oxidizing bacteria (AOB) are obligate aerobes with some species that are tolerant of low oxygen environments [108]. The most common genera of autotrophic ammonium oxidizers are *Nitrosospira* and *Nitrosomonas*, [77,108] which result in the formation of nitrite. AOB are found in most aerobic environments where ammonia is available through the mineralization of organic matter or N compounds are added. Nitrification can also be accomplished by heterotrophic organisms (organisms that require organic matter for energy [102,109]. Heterotrophic ammonia oxidation is accomplished by soil bacteria (e.g., *Arthrobacter globiformis* and *Aerobacter aerogenes*) and most commonly by fungi such as *Aspergillus flavus* [77]. Heterotrophic nitrification appears to be important in soils that are too acidic for autotrophic nitrifiers [110]. The relative importance of autotrophic and heterotrophic organisms for the production of nitrous oxide cannot be readily determined because it is difficult to separate autotrophic and heterotrophic nitrification [111].

Phylogenetic analyses of autotrophic ammonia-oxidizing bacteria DNA sequences show that soil nitrifiers are divided into at least seven groups. Mendum and Hirsch [112] found that the composition of the ammonia-oxidizing populations in agricultural soils, analyzed by DNA analyses, were dynamic and changed their activity, population size, and community structure over time in

response to fertilizer application and cultivation. Such studies that utilize the new phylogenetic analyses have not yet determined the effect of these changes on NO and N₂O production.

In autotrophic nitrification, ammonia (or ammonium) is oxidized to nitrite and nitrate in a two-step reaction where AOB are responsible for the first step and *Nitrobacter* spp. [113] performs the second. Nitrous oxide appears to be formed by a reductive side reaction involving nitrite [105]. Heterotrophic microorganisms oxidize ammonium to nitrite or nitrate in the presence of oxygen and an organic substrate, and they appear to be important in soils that are too acidic for autotrophic nitrifiers [110]. The relative importance of autotrophic and heterotrophic organisms for the production of nitrous oxide cannot be readily determined because it is difficult to separate autotrophic and heterotrophic nitrification [111]. In well-aerated soils, autotrophic nitrification appears to be the main production mechanism for nitrous oxide, but in poorly aerated, nitrate-rich soils significant emissions result from denitrification [114].

Potentially, two nitrogenous gases may evolve from nitrification, NO and N₂O. In well-aerated systems, where NO can be swept quickly from the soil, NO evolution may be 10–100 times larger than that of N₂O [115]. Nitrous oxide evolution becomes relatively more important as the moisture content of the soil increases. This probably occurs because (1) the residence time of NO in soil is longer because of decreased diffusion, which increases the potential of NO to be converted to other products and (2) the activity of enzymes within ammonia oxidizers that reduce nitrite to N₂O are favored in less oxic conditions (fig. 17.1). The nitrifiers are active over a wide range of temperatures (2–40°C) (35.6–104°F), and the optimum pH lies between 7 and 9 [77,113].

The overall nitrification process is controlled primarily by ammonium and oxygen concentrations. Oxygen supply is moderated by soil moisture, and in the normal soil moisture range, the effect of soil water content on N transformations probably reflects its effect on oxygen diffusion (fig. 17.1). The total amount of N lost directly through nitrification is generally minor in terms of N use efficiency, except in specific instances where NO emissions may be large [115].

17.2.3.3 Chemodenitrification

Although biological denitrification may represent one of the major pathways for gaseous N loss, there is evidence that nitrite produced by nitrifying or denitrifying microorganisms can also react chemically to form gaseous N compounds via chemodenitrification [116]. In well-aerated, unfertilized soils, oxidation of nitrite to nitrate by *Nitrobacter* spp. generally proceeds faster than the conversion of ammonium to nitrite, and normally only trace levels of nitrite would be expected to be present (<1 µg N g soil⁻¹). However, high nitrite concentrations are found (>250 µg N g soil⁻¹) when fertilizers such as urea, ammonium carbonate, diammonium phosphate, urea ammonium phosphate, and anhydrous NH₃, which form alkaline solutions upon hydrolysis, are banded in soils. The final gaseous products of chemodenitrification include N₂, N₂O, and NO. While it has not been possible to identify specifically N emissions originating from chemodenitrification in the field, substantial gaseous losses from fertilizer N have been attributed to chemodenitrification in laboratory studies under conditions commonly encountered in the field [107].

Venterea and Rolston [117] developed a model that describes microbial oxidation of ammonium to nitrite and the abiotic decomposition of nitrous acid to form nitric oxide and N₂O (fig. 17.4). Their studies suggest that some degree of transient nitrite accumulation following ammonium application is a consequence of the nitrification process [118]. They found that production of NO and N₂O was highly correlated with concentrations of HNO₂ that originated from autotrophic microbial oxidation of ammonium to nitrate. Production of NO was not, however, correlated with ammonium or nitrate concentrations in the soil or with the overall rate of nitrification.

17.2.3.4 Biological Denitrification

Biological denitrification is the dissimilatory reduction of nitrate and nitrite to produce NO, N₂O, and N₂ by a taxonomically diverse group of bacteria. These bacteria synthesize a series of reductases

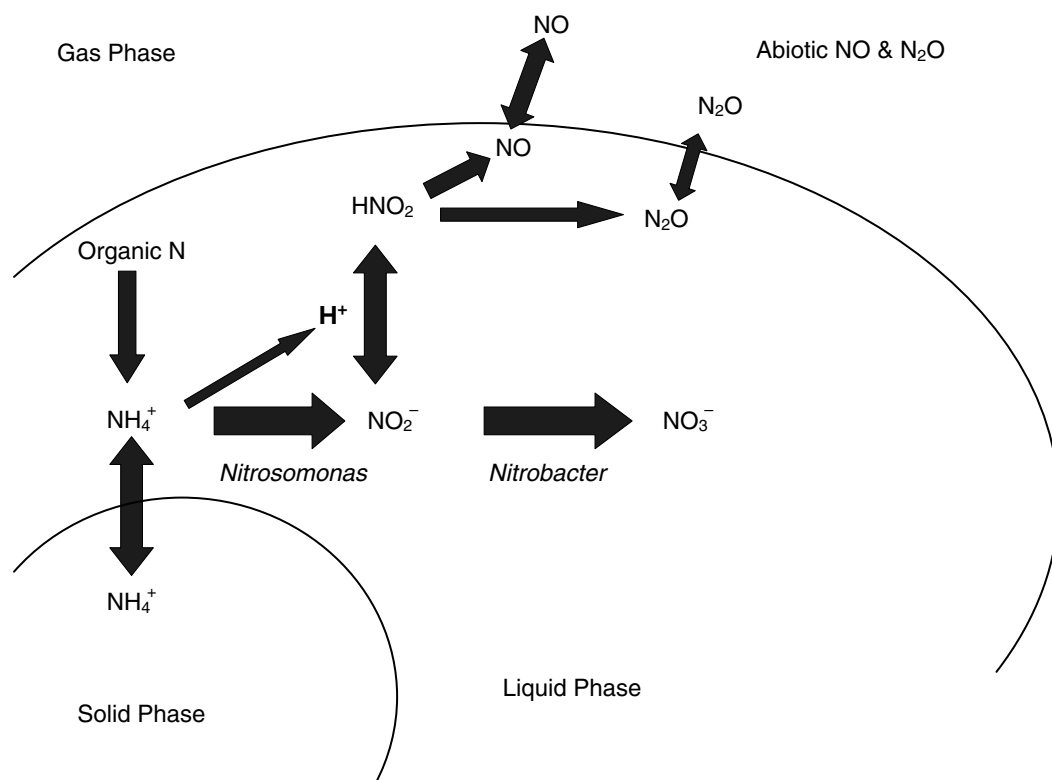


FIGURE 17.4 Conceptual model of microbial nitrification and abiotic production of NO and N₂O (adapted from Venterea and Rolston [117,118]).

that enable them to utilize successively more reduced N oxides as electron acceptors in the absence of oxygen [119]. The general reductive sequence is shown in figure 17.1. In addition to the free living denitrifiers, symbiotically living Rhizobia in root nodules of legumes are able to denitrify nitrate and produce nitrous oxide [120].

The abundant denitrifiers are heterotrophs, which require sources of electron-reducing equivalents contained in available organic matter. Soil factors that most strongly influence denitrification are oxygen (which is controlled primarily by soil water content), nitrate concentration, pH, temperature, and organic carbon. The reductive enzymes are repressed by oxygen but not by ammonium. Nitrous oxide reductase appears to be more sensitive to oxygen than either nitrate or nitrite reductase. Therefore N₂ production predominates in more anoxic sites and N₂O production may be greater in more aerobic conditions. However, the ratio of N₂ to N₂O emitted may also be affected by high nitrate concentrations and associated higher levels of electrical conductivity and osmotic stress [119,121,122] and soil pH (low pH favors N₂O production). Soil water tends to moderate oxygen diffusion in soil and, generally speaking, denitrification occurs only at soil water contents >60% of water-filled pore space (fig. 17.2). Soil organic matter provides energy for denitrifier growth as well as supplying protons and electrons for the reduction process.

Efforts to predict loss of N by denitrification have had limited success because of the interactions of the various factors controlling denitrification. It is presumed that denitrification occurs in soil aggregates and anaerobic microsites, which are heterogeneously distributed in the soil [123]. This may explain the large variation in rates of emission often detected in field studies. The other important variables, which regulate denitrification, pH, and temperature, are generally not the principal constraints in soils where crops are growing because plants have similar pH and

temperature requirements. However, these two parameters may be responsible for limiting maximum rates of denitrification [124].

Groffman and Tiedje [125] suggest that relationships between denitrification and soil porosity vary with soil texture, and that relationships between respiration and porosity vary with soil drainage class. Such concepts can provide a useful rule of thumb when one attempts to broadly predict N loss by denitrification in a particular field. Meisinger and Randall [126] developed a set of estimates of denitrification based on the assumption that oxygen concentration is the major factor influencing losses in the field and that the oxygen is controlled primarily by soil water content. These estimates and direct field estimates of total denitrification losses may amount to <1 to around 100 kg N ha^{-1} in different cropping and grassland systems [107]. Of the total gaseous losses associated with denitrification, only a small proportion (<0.1 – 7% of fertilizer N applied) is generally detected in the form of N_2O [127]. The potential for high denitrification losses appear to be greatest under wet conditions, with very high rates of fertilizer application, or high organic C and N inputs. Data from fertilized perennial ryegrass pastures in Northern Ireland suggest that significant rates of denitrification ($>1 \text{ kg N ha}^{-1} \text{ d}^{-1}$) occur when soil nitrate concentrations in the top 10 cm of the soil profile were above 2 mg kg^{-1} , soil moisture was $>70\%$ water-filled pores, and soil temperature was above 4°C [107].

Research during the past several decades has improved our understanding of how NO and N_2O are produced, factors that control production, source/sink relationships, and gas movement processes. However, despite extensive knowledge of the processes involved, we are only beginning to be able to predict the fate of a unit of N that is applied or deposited on a specific agricultural field (e.g., Davidson et al. [106]; Del Gosso et al. [128]; Frohling et al. [129]; Li et al. [130]; Plant and Bouman [131]; Potter et al. [132]). Studies of emissions of NO_x and N_2O from presumably similar agricultural and natural systems show highly variable results in both time and space. The complex interaction of the physical and biological processes involved must be understood before reliable predictive capability can be developed [3].

17.3 CROP PRODUCTION AND TRACE GAS EXCHANGE

17.3.1 RELEVANCE OF SOIL CO_2 MEASUREMENTS

17.3.1.1 Soil C Change

While terrestrial systems represent a major source of CO_2 to the atmosphere, measurement of soil CO_2 fluxes alone are not necessarily indicative of atmospheric loadings. Net CO_2 flux to the atmosphere from terrestrial systems represents the balance between C inputs by autotrophic fixation and outputs by heterotrophic oxidation of organic material. On a global scale these terrestrial processes are estimated to be roughly in balance [7]. However, at the scale at which management is exerted, there does not necessarily have to be a balance between C inputs and C losses. Systems that are aggressively tilled or where residues are removed may show net declines of soil organic matter over time, and hence are net sources of CO_2 to the atmosphere. No- or reduced-tillage systems, or systems receiving additional C inputs, may show increases in soil organic C and will be net sinks of atmospheric CO_2 . Measurement of soil CO_2 flux will not distinguish net CO_2 flux to the atmosphere. Net CO_2 flux to the atmosphere can be determined from changes in storage. However, small annual changes, coupled with high spatial variability, usually restricts such measurements to long-term monitoring. As indicated in section 17.1.1, there have been several long-term experiments documenting soil C changes.

17.3.1.2 Ecosystem Processes

Micrometeorological methods such as Bowen Ratio and Eddy covariance methods offer opportunities for determination of annual net ecosystem exchange of CO_2 . Within the past decade several flux

monitoring networks have been established to assess net CO₂ exchange from terrestrial systems [133,134]. FLUXNET is a global network of micrometeorological flux measurement sites, which measure the exchanges of carbon dioxide, water vapor, and energy between the biosphere and atmosphere [133]. Over 140 sites on five continents are operating on a long-term and continuous basis. Vegetation under study includes temperate conifer and broadleaved (deciduous and evergreen) forests, tropical and boreal forests, crops, grasslands, chaparral, wetlands, and tundra. It should be noted that the distribution of sites among the different biomes is not equal. These efforts have been primarily focused on forest, grassland, and rangeland systems, however; crop lands have been underrepresented.

The fluxes provided by micrometeorological techniques can provide a direct estimate of net ecosystem exchange of CO₂. It has been demonstrated that indirect estimates of gross primary production and ecosystem respiration can also be obtained [135]. In investigations of seasonal CO₂ flux from a variety of ecosystems, these studies demonstrate that differences in the temporal patterns of assimilatory and respiratory processes is an important reason for characterizing these different components of net ecosystem exchange. Indeed, Falge et al. [135] state,

Hence these drivers (*temperature, moisture, light*) will affect net ecosystem carbon exchange (F_{NEE}) differently as the force F_{GPP} (*gross primary production*) and F_{RE} (*ecosystem respiration*) differently.... Consequently, we need to understand primarily the factors that influence seasonality of the component fluxes, F_{RE} and F_{GPP} and govern the seasonal patterns of net fluxes. [*italics added*]

Since ecosystem respiration is the sum of autotrophic respiration and heterotrophic respiration, similar logic dictates that driving factors may differentially control the temporal dynamics of these two processes. This argues for detailed characterizations of these heterotrophic and autotrophic respiratory processes in order to provide a better understanding of feedbacks on net ecosystem C exchange.

17.3.2 FLOODED RICE

Rice is typically a large part of tropical food production, especially in Asia. In 1995–1997 the global rice production area was approximately 154 million ha, to which 15.4 Tg of N was applied as fertilizer [29]. Although generally considered a tropical crop, rice is grown as far north as 50°N and as far south as 40°S [136]. Because of the unique nature of rice production, typically flooded soils and relatively high N input, the potential for significant emission of CH₄ during flooded periods and N₂O emissions during nonflooded periods exists.

Permanent flooding favors the formation of large amounts of methane whereas even short periods of soil aeration significantly reduce emission rates. Unstable water supply is generic to rainfed rice, so that this rice ecosystem is, in most cases, characterized by lower emission potentials than irrigated rice [137]. In vast parts of equatorial Asia, rainfed rice suffers from dry periods either at the beginning or at the end of the growing season, which reduces the overall emissions by ~50% [138]. However, ample and evenly distributed rainfall may create soil conditions comparable to irrigated rice in some rainfed systems; for example, the rainfed season in Eastern India yielded equally high emission levels as irrigated rice [139].

Conceptually, irrigated rice is characterized by control of the water management, which implies the ability to flood and drain the field whenever it is deemed appropriate from agronomic considerations. In practice, however, irrigation schemes have only a limited buffer capacity against variability in rainfall. Irrespective of the rice ecosystem, it is literally impossible to drain fields at the peak of the rainy seasons, whereas water shortages may occur in some dry seasons in irrigated rice as well. Superimposed on this natural variation of the water regime in irrigated rice are the diverging flooding patterns applied in different regions and seasons. Permanent flooding of rice fields over the entire annual cycle is found in some remote parts of Central China and leads to

extremely high emissions of approximately $900 \text{ kg CH}_4 \text{ ha}^{-1} \text{ yr}^{-1}$ [140]. Consistent flooding throughout the growing season, which is relatively common for the wet season crop in wide parts of Southeast Asia, also entails a relatively high emission potential. Numerous field studies under this type of flooding indicate an emission potential ranging from less than 100 up to $500 \text{ kg CH}_4 \text{ ha}^{-1}$.

In many rice growing regions of China, the flooding of the fields is interrupted by short drainage periods in the middle of the growing season. Although the reduction effect varied considerably at different locations and in different seasons, this local practice reduced emission rates in most cases between 20 and 40% as compared to permanent flooding. In Northern India, irrigation has to compensate for high percolation losses, so that the floodwater is replenished by frequent flooding pulses, for example, once a day [141]. Methane emissions are generally below $30 \text{ kg CH}_4 \text{ ha}^{-1}$ from this type of irrigated rice field. The second management factor determining the level of emission rates is the dose of organic inputs [89,137,142]. Traditional agriculture in China encompasses relatively high amounts of manure leading to high emission rates. The decline of this practice over the last decades has subsequently led to a major reduction of the methane source strength of Chinese rice fields [143]. In addition to exogenous organic material such as animal manure, methane emissions are influenced by the management of crop residues (i.e., straw as well as stubble and roots). Soil incorporation of rice straw generally stimulates emissions, but the incremental effect depends on timing of the straw application. The practice of straw addition is rather unpopular among farmers that have access to other fertilizers, so that the remaining plant parts in the field represent the only input of organic material into the soil. Under these conditions of low organic inputs, even the height of the stubble can have a major impact on the level of emission rates [137].

New emission models and GIS databases may in the future narrow down the enormous uncertainties implied in recent estimates of methane source strengths. Initial approaches for upscaling emissions through coupling of emission models and GIS are shown in Matthews et al. [144]. The MERES model (Methane Emissions from Rice Ecosystem) that was developed by Matthews et al. [144,145] is largely based on a crop yield model (CERES) that was extended by a module describing the steady-state concentrations of methane and oxygen in the soil. The combination with a newly compiled GIS database on rice ecosystems, soils, and weather allowed computation of national source strengths under different crop management scenarios [144]. The baseline scenario assuming no addition of organic amendments and permanent flooding of the fields during the growing season yielded CH_4 emissions of 3.73, 2.14, 1.65, 0.14, and $0.18 \text{ Tg CH}_4 \text{ yr}^{-1}$ for China, India, Indonesia, Philippines, and Thailand, respectively. Scaling up from the 70% of rice production area that these countries represent, a global CH_4 emission from rice fields is an estimated $9.3\text{--}25 \text{ Tg CH}_4 \text{ yr}^{-1}$. From data presented during an international symposium on CH_4 emissions from rice-based agriculture, Sass et al. [12] concluded that current global annual CH_4 emissions from rice fields are likely in the range of $11.9\text{--}16.9 \text{ Tg yr}^{-1}$, which is considerably lower than the latest 5 estimate of $\sim 40 \text{ Tg yr}^{-1}$.

17.3.3 ANNUAL EMISSIONS OF CH_4 , N_2O , AND NO FROM RICE-WHEAT CROPPING SYSTEMS

Relatively few studies have quantified annual fluxes of CH_4 and N_2O or NO in rice-based cropping systems. In many rice-based agricultural areas one or two rice crops and an upland crop are grown. Between cropping periods are fallow times when no crops are grown. Since the climate in much of the rice growing area of the world is semi-tropical or tropical, temperatures are warm during non-rice production periods and when precipitation occurs potentially important N_2O emission events occur during nonflooded times of the year. The rice-wheat crop sequence is becoming more evident in various parts of Asia [140].

Robertson et al. [43] show that to evaluate greenhouse gas emissions from agricultural systems, total global warming potential (GWP) needs to be considered. For rice-based systems a consideration of both CH_4 and N_2O is relevant. Of the few studies that have quantified CH_4 and N_2O emission

TABLE 17.1
Methane and N₂O Emissions in a Rice–Wheat Rotation
in Southeastern China [149,150]

Year 1	+N			0–N		
	CH ₄	N ₂ O	GWP	CH ₄	N ₂ O	GWP
Rice	101	1.95	791	101	0.7	690
Wheat	0	9.79	790	0	3.2	258
Year 2						
Rice	101	3.0	876	101	0.8	698
Wheat	0	12.1	977	0	8.1	654

Note: kg CH₄ ha⁻¹; kg N₂O ha⁻¹; GPW = CH₄ × 23 + N₂O × 296 kg CO₂ –C equivalents ha⁻¹).

through whole annual cropping sequences, two sets of studies, one conducted at the International Rice Research Institute (IRRI) in the Philippines, in rice–fallow–rice–fallow and rice–fallow–wheat–fallow cropping sequences [146–150] and the other at the Wuxian Institute for Agricultural Sciences, Jiangsu Province, China [149,150]. Both studies used automated chamber systems that permitted several flux measurements per day on each location throughout several years of measurement. The Zheng et al. studies are used as an example to demonstrate the interplay between crop management and exchange of different trace gases (table 17.1).

The field plots were fertilized with 191 kg N ha⁻¹ for each crop, using split applications of urea/ammonium bicarbonate fertilizer. During the rice growing season fields were drained mid-growing season of each year. Methane emissions, observed only during the rice growing period, ranged between 42 and 216 kg ha⁻¹ and averaged 101 kg ha⁻¹ during both years. Consumption of CH₄ when soils were aerobic was not observed. Nitrous oxide emissions were observed following midseason drainage but not when fields were flooded. Nitrous oxide emissions totaled 2.0 and 2.7% of N applied during years one and two, respectively. Nitric oxide emissions totaled 1.1 and 1.5% of fertilizer N applied during the same years. Assuming that input of carbon into the soil from the crops was balanced by soil respiration and plant decomposition to CO₂, the net GWP during the rice and wheat growing seasons were not different, with both CH₄ and N₂O contributing during the rice season and only N₂O contributing during the wheat season.

From studies in the Philippines, the GWP from cumulative amount of CH₄ or N₂O (expressed as g CO₂ equivalent m⁻² per season) emitted during each rice production [146] or fallow season [147], from CH₄ emissions generally exceeded GWP from N₂O from continuously flooded rice. Incorporation of rice straw increased CH₄ emissions during rice cropping but had little effect on N₂O emissions. When rice fields were drained, at midtillering particularly, CH₄ emissions substantially decreased while N₂O emissions increased. A side-by-side comparison of the effect of mid-season drainage on trace gas emissions showed that total GWP changed little compared to continuously flooded rice. Total GWP for CH₄ + N₂O was 1040 g CO₂ equivalents m⁻² for the drained plots compared to 800 for the continuously flooded plots. In terms of GWP, the increase in N₂O emissions more than offset the decrease in CH₄ emissions with field drainage [151].

17.3.4 TRACE GAS EXCHANGE IN TEMPERATE AND TROPICAL UPLAND CROPS

There are relatively few measurements of CH₄, N₂O, or NO fluxes in upland tropical crop production systems published (as noted in Bronson et al. [146,147] and even fewer studies where simultaneous

measurements of these gases are reported. Trace gas flux data from temperate cropping systems are more numerous, but reports that present multiple gas exchange are limited. As a result, it is difficult to assess the relationship of the main regulating factors, for example, crop, weather pattern, soils, and fertilization, on total GWP from trace gases in many agricultural systems. Because of the paucity of data we discuss individual gases and describe measurements that have been published during production of various crops.

17.3.4.1 Sugar Cane

Sugar cane is a crop that is grown mainly in the tropics and subtropics. Globally, approximately 16 million ha of sugar cane was grown in 1995 [29], to which about 2.02 Tg of synthetic fertilizer N was applied. The relatively few measurements of CH_4 , NO_x , and N_2O flux in sugar cane fields indicate that fertilizer placement and crop residue management impact trace gas emissions. Weier et al. [154] in northeastern Australia found that the presence of a cane trash layer ($\sim 10 \text{ Mg ha}^{-1}$) tended to increase N losses. N lost from total denitrification ($\text{N}_2 + \text{N}_2\text{O}$) during an 8-day period following application of 160 kg N ha^{-1} of urea-N was 9.2 and 7.6 kg N ha^{-1} with and without the trash layer, respectively. Of this total denitrification, 2.8 and 1.9 kg N ha^{-1} was emitted as N_2O , respectively. In another study, Weier [155] found high rates of CH_4 oxidation, $0.7\text{--}2 \text{ kg CH}_4\text{-C ha}^{-1} \text{ d}^{-1}$. The highest oxidation rates were from plots that had been fertilized with KNO_3 (160 kg N ha^{-1}). CH_4 uptake rates with a trash layer were generally higher than without trash, which Weier [155] attributed to retention of soil moisture by the trash layer. In other experiments in Australia the effect of trash layer on fertilizer N losses were variable from year to year and within different soil types [156] and ranged from 4–65% of fertilizer N applied.

Matson et al. [157] observed the influence of soil type and N-fertilizer application method on sugar cane fields on NO and N_2O emissions in two locations in Hawaii. Subsurface drip irrigation/fertilization in Mollisol and Inceptisol soils in Maui was compared to surface broadcast application of fertilizer N in Andosols in Hawaii. Subsurface fertilization decreased NO emissions compared to surface application but increased N_2O emissions. N_2O fluxes were lower from the Mollisol soils compared to Andosol soils at the same soil WFPS. Seasonal NO + N_2O emissions totaled 0.03–0.5% of fertilizer N applied in drip irrigation systems and 1.1–2.5% when surface application of urea was practiced.

17.3.4.2 Cotton, Maize, and Wheat

Based on the few published studies of trace gas fluxes from tropical upland production of cotton, maize, sorghum, and wheat, fertilization (nitrate and ammonium availability) and soil moisture are the dominant controls on N_2O and CH_4 fluxes. The same can be said for temperate systems as well (e.g., Dobbie et al. [158]; Flessa et al. [159]). Because these upland systems are better drained, CH_4 fluxes are low compared to flooded crop systems.

Mahmood et al. [160,161] quantified fertilizer N loss (based on ^{15}N recovery) and total denitrification (using acetylene block techniques) in irrigated cotton, maize, and wheat in a semiarid subtropical region of Pakistan. They found that total fertilizer N losses were typically 30–45% of the N applied and that denitrification accounted for 3–40% of the loss. Neither N_2O nor NO_x fluxes were measured individually. The authors considered NH_3 volatilization to be a main loss mechanism when denitrification was limited. The high denitrification rates in cotton were ascribed to high August temperatures and concomitant large rainfall events. Temperatures were lower and rainfall less abundant during the maize–wheat cropping periods [161].

In an irrigated wheat production system in Sonora, Mexico, Matson et al. [162] found that N_2O and NO_x emissions were large, $\sim 8.5 \text{ kg N ha}^{-1}$, under conventional farming practices for the region. These losses were decreased to $\sim 4.4 \text{ kg N ha}^{-1}$ by instituting alternative fertilization

TABLE 17.2
Estimates of N₂O Emitted During the Maize Growing Season at
Four Locations in Thailand and Two Locations in the U.S.

Location	Not Fertilized	N-Fertilized
	kg N ₂ O-N ha ⁻¹	kg N ₂ O-N ha ⁻¹
Nakhon Sawan, Thailand	0.10	0.43
Phra Phutthabat, Thailand	0.12	0.35
Khon Kaen, Thailand	0.11	0.29
Chiang Mai, Thailand	0.12	0.2
Tennessee	1.9	4.6
New York	0.3	2.5
From [164–166]		

practices, which decreased fertilizer input from 250 to 180 kg N ha⁻¹ while maintaining crop yield. Total fertilizer N losses were 70 and 48 kg N ha⁻¹ under conventional and improved fertilization practice, respectively.

Crill et al. [153] employed an automated chamber system to monitor N₂O emission from a maize field in Costa Rica. They found that N₂O emissions from fertilized (122 kg N ha⁻¹/cropping season) versus unfertilized plots averaged 640 μg N₂O-N m⁻² h⁻¹ versus 120 μg N₂O-N m⁻² h⁻¹. Likewise, Weitz et al. [163] found that fertilized systems in Costa Rica had more than three times the N₂O emissions as unfertilized systems. Both authors observed that highest N₂O fluxes were associated with surface WFPS between 80–99% and that fertilization and soil moisture were the dominant regulators of N₂O flux.

Watanabe et al. [164] measured N₂O emissions from maize fields at four sites in Thailand (table 17.2). They found that the N₂O emission increase due to N-fertilization of 47–75 kg N ha⁻¹ was small, averaging 0.1–0.4% of applied N at four sites. Comparative N₂O fluxes for a cool, humid temperate location, New York [164], and a warm, humid temperate site, Tennessee [166]. Field plots near Ithaca, New York, were fertilized with 0 or 120 kg of urea N while the field located near Jackson, Tennessee, was fertilized with 140 kg of ammonium nitrate and cropped with maize. The warm–humid temperate site emitted almost two times as much N₂O as the cool–humid site. Nitrous oxide emissions were several times greater in both temperate sites than in the tropical fields in Thailand. The timing of rainfall events appear to be a major controller of N₂O fluxes at all of the locations.

17.3.4.3 Impact of Crop on N₂O Emissions in Scotland

From a multiyear analysis of N₂O emissions from intensive cropping systems in Scotland (table 17.3), Dobbie et al. [158] observed that increased flux was associated with increasing soil water-filled pore space, temperature, and mineral N content due to fertilizer application. Fluxes were low when any of these variables was below a critical level. The largest fluxes occurred when WFPS was 70–90%, suggesting that denitrification was the major process responsible. They found that annual emissions varied widely and associated this variation to degree of coincidence of fertilizer application and major rainfall events. Emissions from spring barley and winter were similar (0.3–9 kg N ha⁻¹ yr⁻¹). These were similar to those found in studies in irrigated systems in Colorado [167,168]. Fluxes from potatoes (3.0–4.7 kg N ha⁻¹ yr⁻¹) and broccoli (9.1–12.2 kg N ha⁻¹ yr⁻¹) were considerably larger, but in the range of fluxes reported for potatoes by Flessa et al. [159] in southern Germany (summer fluxes of 1.6–2.0 kg N ha⁻¹) and Ryden and Lund [169] (in irrigated vegetable production in California (20–40 kg N ha⁻¹).

TABLE 17.3
Effect of Crop on N₂O Emissions

Crop	kg N ₂ O-N ha ⁻¹ yr ⁻¹		% N Applied	Crop Years
	Average N ₂ O Flux	Range		
Spring barley	0.8	0.7–0.8	0.7	3
Winter wheat	0.8	0.3–0.9	0.4	4
Potatoes	4.0	3.0–4.7	2.4	3
Broccoli	10.7	9.1–12.2	6.2	2

Adapted from Dobbie et al. [158]

17.4 IMPACT OF TILLAGE ON N₂O, CH₄, AND SOIL ORGANIC CARBON IN CROPPED SOILS

No-till (NT) management has been promoted as a practice that offsets the global warming potential (GWP) from emissions of N₂O and CH₄ in crop production because of its ability to sequester carbon in the soil [6]. In a recent analysis of available field data, Six et al. [170] found that newly converted NT systems increased GWP relative to conventional tillage practices in both humid and dry climates (table 17.4). In this analysis GWP is defined as the sum of the difference in N₂O, CH₄, and soil organic carbon (SOC) between NT and CT, expressed in CO₂-C equivalents. Only in humid climates did longer maintenance of NT, >10 years, significantly reduce GWP. Mean, cumulative GWP over a 20-year period is also reduced under continuous NT in dry areas. Emissions of N₂O drive much of the trend in net GWP. The limited data sets available for such analyses accentuate the high uncertainty associated with the N₂O flux data, thus in the interpretation of the data. The decrease in N₂O flux over time that a system has been in NT is attributed to increased soil aggregation and improved aeration status. During the first few years of NT, soil bulk density in the top 30 cm may increase, but as SOC accumulates over time, soil structure improves as more stable aggregates develop. Soil aeration improves concomitantly [170]. The resulting effect is a decrease in net GWP over time in dry and humid climate soils.

A very different picture of the effect of time that a system has been in NT on GWP is given in a modeling study [171]. Using the DAYCENT ecosystem model, they observed that during the first few years of NT, soil may decrease net GWP and over time, as the rate of increase in SOC declines and N₂O emissions increase, the net GWP increases. Simulating U.S. Great Plains cropping systems, baseline soil conditions were developed using 100 years of historical land use, followed by 50 years of improved management (i.e., conversion to NT). The 50 years were divided into 12-year segments and the change in SOC after each 12 years was assumed to represent net C exchange between the atmosphere and the soil–plant system over that 12 years. The estimated soil-atmosphere exchange of N₂O and CH₄ over each 12-year period was converted to CO₂-C equivalents and assumed to have 311 and 21 times 16 the GWP of CO₂ on a per molecule basis. Production of each gram of N fertilizer was assumed to require 0.8 g of CO₂-C. Net greenhouse gas was calculated by summing the change in SOC and the CO₂-C equivalents of N₂O, CH₄, and N fertilizer production (table 17.5).

This simulation suggests that the impact of NT on net GWP decreases over time in a dry agroecosystem. During the first 12-year period, the change in SOC is greatest and N₂O emissions are lowest [171]. Over time, the rate of C-sequestration declines and N₂O emissions increase because the rate of immobilization of inorganic N declines. The model predicts that the small soil CH₄ sink declines under NT because of higher soil water content. Changes in CH₄ consumption are small, however, and have little impact on net GWP estimates, as demonstrated by a Robertson et al. [43] field study. The DAYCENT model does not account for changes in soil structure following conversion to NT but does allow uniform conditions for all comparisons. The data used

TABLE 17.4
Annual Differences in Soil-Derived Greenhouse Gas Fluxes and Global Warming Potential (GWP) Between No-Till and Conventional Till Systems

No-till minus Conventional till (CO ₂ -C equivalents kg ha ⁻¹ yr ⁻¹)											
Year 5				Year 10				Year 20			
Soil	kg ha ⁻¹ yr ⁻¹		GWP	kg ha ⁻¹ yr ⁻¹		GWP	s.e.	kg ha ⁻¹ yr ⁻¹		GWP	s.e.
	Estimate	s.e. ^a		Estimate	s.e.			Estimate	s.e.		
Organic C	53	1.1	-194	4.4	0.5	-213	1.9	61	0.3	-222	1.1
	-83	1.6	306	5.7	0.8	37	2.7	26	0.5	-97	1.6
N ₂ O	1.0	0.2	304	65	0.2	90	61	-1.1	0.5	-338	154
	0.4	0.4	109	117	0.3	73	100	0.0	0.4	8	127
CH ₄	-0.2	0.03	-3.5	0.8	0.03	-3.5	0.8	-0.2	0.03	-3.5	0.8
	-0.2	0.03	-3.5	0.8	0.03	-3.5	0.8	-0.2	0.03	-3.5	0.8
Soil-derived GWP											
Humid			107	65		-126	61			-563	154
Dry			411	118		107	100			-98	127

Note: Negative numbers indicate a reduction in GWP

^a s.e. = standard error and GWP units are CO₂-C equivalents (kg ha⁻¹ yr⁻¹). Values in the columns headed by year 5, 10, and 20 are estimates for 5, 10, and 20 years after conversion from conventional till to no-till. Estimates are based on output of linear mixed-effect modeling of available data. Humid and dry climate designation is based on Holdridge Life Zones [182], which classify areas with a potential evaporation/mean annual precipitation ratio >1 as dry and areas with a ratio <1 as humid climates.

Adapted from Six et al. [170]

TABLE 17.5
Simulated Changes in Net GWP for Consecutive 12-Year Periods
Following Implementation of No-Till in the State of Kansas in
the U.S. Great Plains

Years after converting to no-till	Net GWP in CO ₂ -C equivalents
0–12	–800
12–24	–300
24–36	–100
36–48	+300

From DelGrosso et al. [171]

TABLE 17.6
Trace Gas Fluxes Observed in Four Field Studies in
Conventionally Tilled (CT) and No-Till (NT) Soils

Location	Tillage	g N or C ha ⁻¹ d ⁻¹	
		N ₂ O	CH ₄
Oxfordshire, England	NT	7.0**	nd*
		3.3	nd
	CT	1.8	nd
		0.8	nd
Sydney, Nebraska	NT	1.2	–8.1
	CT	1.4	–8.0
Quebec, Canada	NT	9.6**	nd
	CT	6.0	nd
Hickory Corners, Michigan	NT	3.2	–1.6
	CT	3.1	–1.7

* nd = not determined
** significant difference between NT and CT (*p* < 0.1)

Data from Robertson et al. [43], Kessavalou et al.[172], MacKenzie et al. [173], Burford et al. [174].

in the Six et al. [170] evaluation are, in contrast, from a variety of studies that were likely conducted using a variety of methodologies. As a result, the relative importance of improved soil structure on decreasing N₂O emission suggested by the array of field studies and the DAYCENT model projection of increased N₂O emissions over time cannot be evaluated at this time.

As the Six et al. [170] analysis indicates, the system response may differ by climate and cropping practices within NT systems. An example of the diversity of response of trace gas exchange to NT is drawn from four field studies (table 17.6). These studies were conducted in temperate semiarid western Nebraska [172]; temperate cool–humid Quebec, Canada [173], Oxfordshire, England [174]; and central Michigan [43].

Nitrous oxide fluxes were higher in NT than in CT in the studies in England and Canada but were not different in the Nebraska and Michigan studies. Soil moisture was likely continually higher at the sites where N₂O emissions were higher in NT, but these sites had been converted to NT less than 10 years before the studies were conducted. Tillage had little effect on soil CH₄ consumption or N₂O emissions in the semiarid wheat-fallow system in Nebraska or the crop rotation studies in more humid Michigan. These sites had been converted to NT 20 and 10 years, respectively, before the gas flux measurements were made.

17.5 IMPACT OF SOIL FREEZE/THAW ON N₂O FLUX

Annual N₂O emissions are frequently enhanced in climate zones where soils freeze and thaw (e.g., Cates and Keeney [175]; Goodroad et al. [176]; Kaiser and Ruser [177]; Lemke [178]; Wagner-Riddle [179]. Such studies indicate that 15–75% of annual fluxes arise during freeze–thaw periods in the winter and early spring time where these events occur. In these systems, cropping season N₂O fluxes are typically related to N input and soil WFPS [152]. Lemke et al. [178] observed that N₂O emitted during spring that represented almost 60% of annual emissions in fertilized winter wheat and fallow fields and about 30% in an unfertilized wheat field (fig. 17.5). Lemke et al. [178] found that soil nitrate concentration and WFPS explained 78% of the variability of N₂O flux. They also observed that soil nitrate concentration measured in the autumn explained almost 50% of the variability of N₂O emissions at spring thaw. Kaiser and Ruser [177] observed, from an evaluation of six long-term field experiments in Germany, that winter N₂O fluxes showed no relationship to growing season fertilizer application rates. Wagner-Riddle et al. [179] winter/spring N₂O emissions near Guelph, Canada, ranged between 0 and 4.8 kg N ha⁻¹, depending on cropping system. They observed that fallowing, manure application, and alfalfa incorporation in the autumn lead to high spring emissions while the presence of perennial plant cover appeared to nearly eliminate emissions during spring thaw.

17.6 CONCLUDING REMARKS

Production of trace gases (CO₂, CH₄, N₂O, and NO) in and emission from the soil from diverse agricultural productions are performed by basically the same processes. Net CO₂ flux to the

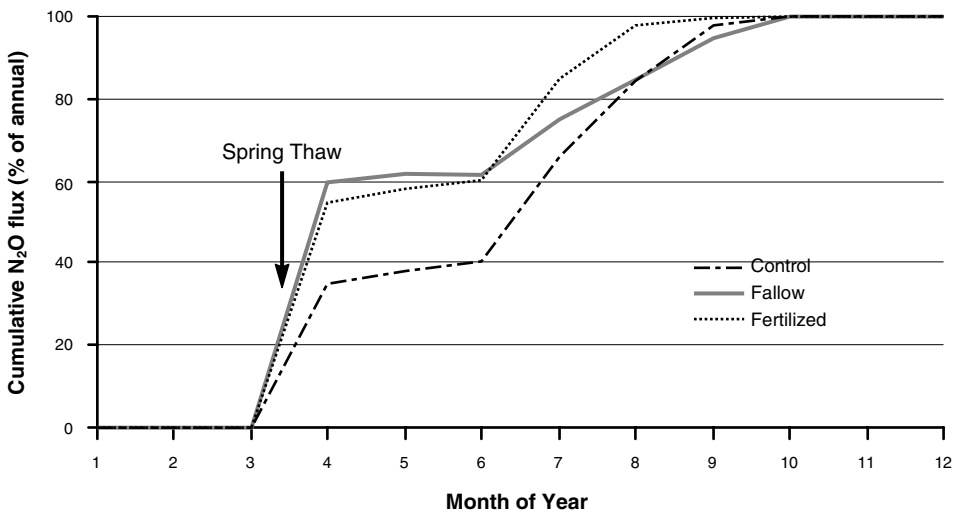


FIGURE 17.5 Seasonal distribution of N₂O emissions from field sites located near Lethbridge, Canada (adapted from Lemke et al. [178]).

atmosphere from terrestrial systems represents the balance between C inputs by autotrophic fixation and outputs by heterotrophic oxidation of organic material. On a global scale these processes are estimated to be in balance. Historically, it has been well documented from several long-term studies that agricultural practices have shifted the balance between CO₂ fixation and respiration, resulting in a net loss of soil organic C, due to accelerated decomposition processes (see Paul et al. [8] for summaries and accompanying data). This trend can be reversed, however, by increased crop biomass production and reduced soil disturbance.

Agricultural soils that produce CH₄ are primarily flooded rice fields, although small emissions have been observed in a number of upland crop production systems [174]. Although generally considered a tropical crop, rice is much more diverse and is grown as far north as 50°N and as far south as 40°S [136]. Methane emissions across this diverse region are ubiquitous and dependent upon organic substrate, oxygen-limited conditions, and temperature [137]. Unique microbial characteristics may exist between different rice fields, but are unknown at this time. In upland cropping systems, N₂O is typically the main trace gas of interest. Nitrous oxide and nitric oxide are produced through nitrification/denitrification and are principally the same whether in rice fields in the tropics or vegetable fields in Scandinavia. Chemoautotrophic bacterial nitrification is a process mediated by a relatively limited array of bacteria that lack a pronounced diversity, but the process occurs in essentially all terrestrial, aquatic, and sedimentary ecosystems. Denitrification, on the other hand, is performed by a diverse and widely distributed group of aerobic, heterotrophic bacteria that are facultative anaerobes. The general requirements for denitrification are the presence of bacteria possessing the metabolic capacity, suitable electron donors such as organic C compounds, reduced S compounds or molecular hydrogen, anaerobic conditions or restricted oxygen availability, and N oxides as terminal electron acceptors [119]. Throughout the presentation in the previous pages one may note that variability of soil moisture and substrate were mentioned as suspected controllers of N₂O and NO emissions. The relationships between soil water-filled pore space and nitrification/denitrification demonstrated by Lin and Doran [67] hold true for most field conditions (fig. 17.2). The relationship drawn by Dobbie et al. [158] demonstrate the nature of soil water controls on denitrification and N₂O emissions that are applicable across a diverse array of agricultural fields.

Fluxes of N₂O and NO are frequently reported to be influenced by soil water content, frequently expressed as WFPS, and soil nitrate and ammonium concentrations [37]. Thornton and Valente [166] found that correlation coefficients for N₂O and NO flux with WFPS were 0.32 and 0.14. The individual relationships of such parameters are not always as definitive [167,181], but the typical response is that when substrate is not limiting that N₂O emissions increase with increase in WFPS as described by Dobbie et al. [158] Thornton and Valente [166] observed strong negative correlation between the ratio of NO/N₂O emissions and WFPS in Tennessee.

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18 Principles Behind Order and Sustainability in Natural Successions and Agriculture

Gero Benckiser

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18.1 INTRODUCTION

Agricultural landscapes comprise monocultures, polycultures, mixed crop–livestock systems, agroforestry, agro-silvo-pastoral systems, aquaculture, rangelands, pastures, and fallow land (chapter 1 in this volume). The individual anorganical, organical, and biological parts (chapters 3–17 in this volume) interact with each other in a highly complex fashion (chapters 19–20 in this volume) and can sustain high productivities over centuries, as worldwide distribution from the beginning of agriculture intensively cropped loess areas reveal. Despite farming, loess-based agricultural production systems seem to be surprisingly robust.

The classical strategy to cope with complexity is that of *Descartes*, who recommended to decompose systems step by step into its elementary parts until one arrives at a level at which these

parts can be understood and managed. Molecular biology using the central dogma of storing information as a codescript in nucleic acid structures pursues this line quite clearly. In contrast, observations made in animated and inanimated systems reveal that new qualitative features are brought about to a macroscopic level by means of interacting elements (e.g., the electronic properties and chemical reactivity of small metal clusters can be fundamentally different from bulk metals¹⁶). Evidently, the total is more than the sum of its parts or the world of phenotypes can be (1) real or not, (2) real but indescribable, (3) not real and indescribable, or (4) simultaneously real, not real, and indescribable (Aristoteles or the Indian mathematician Mahavira).⁹⁹ The formation of complex structures from simpler units like atoms, molecules, cells, individuals or societies of bacteria, plants, animals, or human beings occurs often self-organized, meaning that single disordered elements interact with each other in a dynamic process for creating new systems with a new specific order.²³ Input signals going through control circuits are transformed into output signals and channeled into spatially new arranged patterns. Only in very few cases the physico-chemical and/or genetic-biochemical mechanisms are understood and mathematically described. An enormous gap remains in our understanding. The discipline that tries to find explanations for the effects (benefits) of cooperating efforts has been introduced by Haken and Graham⁷⁸ and is called *synergetic* (συν-ενεργεια, working together).^{78,81,82}

Even the description of the individual parts of an ecosystem leads to an enormous amount of information, formulated as

$$H(X) = - \sum_{x \in X} p_i(x) \log_b p_i(x)$$

where X is a discrete random variable, p_i the probability of the i th symbol type in a set of available symbol types, and $\log_b$ the information (I). To simplify the flood of I it has to be compressed.^{81,171,172} The transfer of an original I into a compressed form I' without any data loss is termed “entropy coding” and can be expressed in bits when the logarithm is 2. Entropy coding, a minimal I -length, or a minimal number of bits is achieved by dividing the counted frequency of symbols in the I -sets through the number of repetitive sections. Biological I -compression is illustrated, for example, by a certain length of a DNA sequence that may carry the I either for one or for several proteins. The mechanism behind overlapping genes is called alternative splicing of introns and exons. Its regulation defines the algorithm of I -compression. The real-time view of videos on mobile phones through the reduction of video data by intelligent computer software is another modern application of I -compression. Similarly, intelligent programs are using neuronal network techniques under development on which highly desired early warning systems in ecology and environmental science are based. In principle, this type of I -compression works like human visual recognition, always dividing our field of view into a static and moving part.

Informational entropy describes entropy in the thermodynamical context as the quantity of energy no longer available to do physical work ($S [U, V, N]$; U = inner energy, V = volume, and N = number of particles in the considered system). A number of examples from the inanimated and animated world reveal that small entropy-creating events can mark macroscopical changes. DNA, for example, is most of the time highly ordered and very effectively compressed. At replication events the condensed DNA resolves to optically invisible structures or to new ordered structures like the lampbrush-chromosomes in animals and insects. Systems shattered at changing conditions go over into phase transition and have to decide whether they enter into disorder or go back to stabilization. As the DNA example shows, restabilization may lead to the emergence of a new macroscopic structure of different quality. Phase transitions in inanimated systems are (1) freezing where water goes suddenly over from the liquid into the solid ice phase, (2) the onset of ferromagnetism, or (3) the onset of superconductivity. As a biological example, whales use phase transition of fat from the solid into the liquid form to ease diving. A summation of small identical forces evidently lead to a harmonized working together and finally to system improvements. A brief look into the human

intestinosphere with its about 500–1,000 different species reveals that all these species depend obviously highly on each other, since at present only less than 50% of these species can be cultivated *ex vivo*.^{85,72} Everything seems to influence everything. If the intestinosphere is shattered by a bacteriophagal-initiated infection or a sudden change in nutrition then it goes over into a phase transition, which may end in regained health as the pandemic character of infections or diarrhoea are documenting it.

Microbial population densities as high as in the human intestinosphere are found on plant surfaces in agroecosystems, around or inside of open or clogged soil pores, intercellulars, nodule-like organs, vacuoles, or faunal and grazing animal intestines. Plants with their bacterial endosymbionts, the chloroplasts, and photosynthetically free-living bacteria capture the solar energy on which all others are living after the transformation into chemical and biological energy (adenosine triphosphate, or ATP). Additionally to sun-derived energy, add farmers' energy in the form of fertilizers to their fields. Both energy inputs enable the anabolic soil processes to transform small molecules like N_2 , CO_2 , CH_4 , and H_2O into polymers and living structures, which under ATP gain are again catabolically degraded into small molecules. Catabolic active organisms very rarely consume the energy stored in the polymers completely. Often only one degradation step is carried out. The rest is left for others to live on (chapter 16 in this volume). Soil anabolism and catabolism is often organized in a job-sharing way, as will be discussed in the following sections of this chapter. Water is the transport medium. The capability to store water in the cell or tissue thus predominantly decides the biodiversal sizes within soils (chapter 13 in this volume).

Along with selected examples from the inanimated and the viral, bacterial, protozoal, nematodal, leguminous, ruminant, earthworm, and antal world, this chapter tries to unveil how agroecosystems might recover from perturbations and sustain productivity.

18.2 THE LASER: AN EXAMPLE OF SELF-ORGANIZATION IN THE INANIMATED WORLD

Order–disorder relationships follow the laws of thermodynamics, describing energy transfers (e.g., that of thermic energy, heat) and work. Closed systems are isolated from their environment and their thermodynamical equilibria are characterized by minimum energy and/or maximum entropy or are a compromise of both. Open systems, in contrast, are in contact with their environments by exchanging energy as well as entropy (and sometimes mass). Influxing energy enables them to carry out work and to degrade entropy, measured in joules (J). Order from disorder can develop. Turbulent flows generate new dissipative structures that use eddies inside of eddies to convert energy into lower scales. Dissipating heat causes temperature changes and along a hierarchy of nested eddies small random fluctuations can evidently stabilize and generate new, complex patterns.¹⁴⁸

An energized gas-laser (light amplification by stimulated emission of radiation) is a relatively well-understood example for new pattern formation (fig. 18.1). A central part of a gas-laser is an energizable resonator cavity filled with gas and shielded by two semi-reflecting mirrors. Inside of the laser resonator excited electrons move incoherently from the outer to the inner orbit of the gas molecules by emitting incoherent light. Suddenly further energizations lead to a dramatic change of the statistic light properties of the gas molecules. At the lasing threshold the incoherent light amplitudes (A) go over into coherent electromagnetic waves (modes) with discrete frequencies and very little phase and amplitude fluctuations. The electrons start to organize themselves in a synchronized way. Spontaneous emission organizes the incoherent light and absorption is the mechanism. Absorption brings an electron to a higher energetic level and coherent light develops through forced or stimulated emission. A light wave with its inhabiting frequency and phase Φ hits an already energized electron and forces it to leave the energized status by sending out a light congruent to Φ of the hitting wave. The electron drops to a lower energetic level by doubling the energy of the hitting light wave. The quantum energy of the laser wave $h\nu$ is then $E_2 - E_1$ (h = Planck's

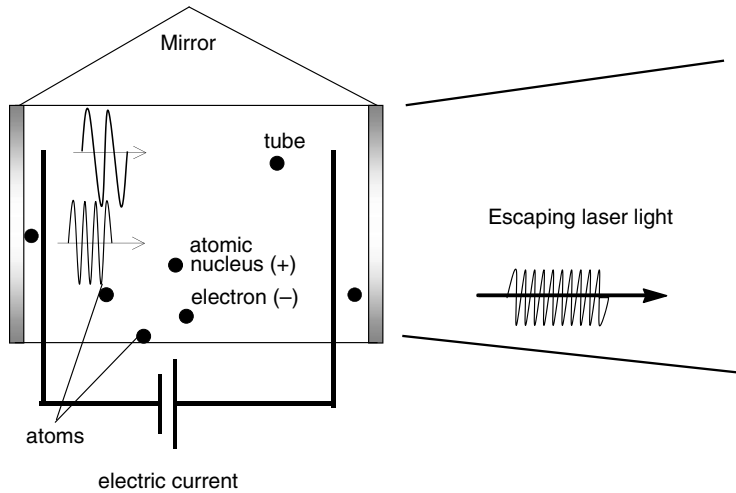


FIGURE 18.1 Typical setup of a gas laser. In a conventional lamp, the light field generated by the gas atoms consists of individual wave trains that represent noise. In a laser consisting of a glass tube with two semi-reflecting mirrors mounted at its end faces, the enclosed gas atoms emit a highly ordered light wave goes through one of the mirrors after they have been excited by an electric discharge. Adapted from Haken.⁸¹

constant = 6.624×10^{-27} erg-seconds; ν = frequency of the lightwave; E_1 = starting, E_2 = final energy). Double energized modes show self-organized amplification effects and start to release coherent light like an avalanche.

During the phase transition a light field intensity E' is emerging. E' is a system-relevant control parameter (attractor) or parameter of order. It determines the behavior of the other parts of the system. The single parts, the many photons of the same frequency and phase Φ , in reverse, feed back on E' and the stimulated emission connects and synchronizes all components (fig. 18.1). A synchronized angular acceleration is observed. E' becomes macroscopically observable in the form of laser light. Order from disorder develops.⁸¹ Highly energized laser light leaves the resonator and interacts with the surrounding environment. Energy dissipation, proportional to the rotation velocity, grows nonlinear with the third potency of velocity. Entropy with the dimension joule K^{-1} develops. The development from incoherent to coherent light in a laser can be mathematically described (equation 18.1, 18.2).⁸¹ The laser wave or mode with the highest coefficient α (equation 18.1) wins. The environmental conditions (dU/dt) control the competition (equation 18.2).

$$dA/dt = (\alpha D_0 - k) \times A - \alpha CA \quad (18.1)$$

where A = light amplitude; t = time; α = constant of absorption or enslaving constant; D = inversion = $N_2 - N_1$ (N_1 = number of nonexcited electrons, N_2 = number of excited electrons); D_0 = inversion without energy input (starting conditions); k = decay constant; C = proportional factor = $W_2 - W_1$ (difference in energy of a electron).

$$dU/dt = N(U, \Delta, \sigma) + F \quad (18.2)$$

where U = dynamic of the actual conditions; t = time; N = nonlinear functions of the actual conditions; Δ = spatial inhomogeneities; σ = changes of the control parameters; F = directed random forces (vector of control parameters).

18.3 VIRUSES: MEDIATORS BETWEEN THE INANIMATED AND ANIMATED WORLD

DNA- or RNA-structures and protein envelops (capsids) differentiate viruses morphologically. Their sizes range from approximately 3,200 nucleotides (Hepadnaviruses) to approximately 800 kilobase pairs (kbp, Mimivirus). Among viruses more genetic diversity is assumed than in all the rest of the animal, plant, and bacterial kingdoms.¹³¹ Viruses are condemned to be obligate intracellular organisms because they lack in metabolism to replicate themselves. All need a host, which can be microorganisms (bacteria, fungi, algae, protozoa), (soil) animals, or plants. Viruses replicating in plants are called retroviruses because they have generally RNA as genome that has to be translated into DNA before reproduction can occur. The smallest viruses are called viroid. They are reduced to free RNA molecules of 250–450 bp in size, but can cause severe economic losses, for example, in coconut plantations.¹⁸⁵ Viruses replicating in bacteria are called bacteriophages, or phages and consist of single- or double-stranded DNA.¹⁰⁹ Protein structures (prions) can as viruses replicate in hosts and cause conformational changes on related proteins, resulting, for example, in bovine spongiforme encephalopathy (BSE).^{52,53}

Viral DNA/RNA or prions are dissipative structures. They exchange genes including the stored information.¹⁴⁸ Assuming a constant abundance of the four bases adenine, thymine, guanine, and cytosine are in the DNA and RNA molecules, then the information content (**I**) is $\log_2 4$ or 2 bits.¹⁷² The stored **I** is redundant or lower than $\log_2 4$ when the bases are repetitive or only one base forms a section of DNA or RNA. Then **I** equals the regularly returning sequence and is lower. **I** is maximal at an unregular order of the four bases.⁸² The 5243 bp of a Simian 40 virus would have an **I** at a regular base pair order of about 10.486 bits ($\lg_2$ multiplied by the base pair number). Actually the double-stranded Simian 40 DNA has certain DNA sections that code for up to three proteins (alternative splicing of introns and exons).⁹³ Thus, its **I**-content is higher than the bp-order suggests. Further, the methylated cytosine that switches genes on and off by methylation and demethylation should be considered as a 5th base.¹¹³ If the **I** stored in proteins is included then the genomal **I** should be considerably higher.²⁰⁰

Viral replication is also considered important in exchanging **I** in nature.¹³¹ It begins with the attachment of the freely existing viral form, the virion, to a host. After the viral nucleic acid has penetrated the host cell the infectivity stops. This phase, called eclipse, persists until new complete virus particles reform after replication. First starts the host DNA to degrade. Then the metabolic cell machinery shifts toward the synthesis of viral proteins and nucleic acids. The viral DNA replicates bidirectionally in the bacterial genom and stays either linear or is circulized by a rolling-circle mechanism. The endproduct is a long, double-stranded DNA with repetitive copies of the viral genome, the concatamer. Occasionally phage assembly and/or maturation can lead to a burst of the host cell in a time window of about 5–10 minutes. This form of viral replication releasing the virus particles into the surrounding environment is called lytic because of its virulence and seems to occur relatively rarely in nature.

The virus particles released, for example, into soils, will now intimately mix with the uptaking matrix and will be low concentrated. Independently of the inhabiting kinetic energy viruses as nonmotile bacteria in a low-concentrated status carry out an irregular, trembling movement. The virus particle density per volume increases through evaporating waterfilms. Then the trembling goes over into a more directed (ordered) movement, called diffusion. Diffusion improves the probability to find a new host cell for a new infection cycle.

The more typical form of viral replication is lysogenic reproduction, where the viral genome stays for an undefined period integrated into the host DNA (e.g., a bacterium). The virus reproducing with the dividing host cell is called prophage. This cointegrated status is fairly stable, because the repressor that controls prophage expression binds efficiently to new phage DNA and anticipates lysis. Mutations

in the integrated viral DNA can lead to an irreversible behavioral change and the viral DNA becomes a permanent part of the host chromosome (e.g., the chromosome of the human pathogenic *Escherichia coli* strain 933W includes 19 defective prophages).¹⁸ Prophages as permanent parts of the host chromosome increase the genome elasticity significantly and host survival improves.¹⁸ It is assumed that lysogenic conversion could be responsible for the production of the *Bacillus anthracis* toxin.¹³¹ Viral genes are often integrated into relatively moveable bacterial DNA forms, the plasmids. Plasmids ease the traveling of the integrated viral gene into a new host by conjugation.¹⁹⁵ Lysogenic conversion is probably also behind the change of the harmless human intestinal *Escherichia coli* strain into a harmful shigella-like toxin carrying enterohemolytic bacterium (EHEC) through the *E. coli* phage W933. EHEC strains, first detected in 1982 in Oregon, survive in cows without causing infection symptoms, but if they reach human beings in milk enterohemolysis can break out. Meanwhile, the EHEC toxin genes carry quite a number of different phages. Places of intensive recombinations in between phages are grasslands, the rumen of cows, and wastewater treatment plants.¹⁸⁶

Lysogeny is occasionally either spontaneously or artificially induced by, for example, chemicals into a lytic cycle. During the conversion phase, called intermittency, the viral DNA is shattered, starts to oscillate, iterate, feed back, and the virus–host relationship changes. The host–virus genome integration is given up and may lead to a persistent (continuous release of viruses from a cell that is not immediately killed), an abortive (viral multiplication is hindered by a defect in needed components), or a transforming infection (cell transformations lead to abnormal cell growth characteristics, e.g., cancer initiated by tumor-causing DNA viruses). There is a dearth of quantitative data on the impact of viral-mediated cell lysis.¹⁸⁵ The nutrients released from lysed cells are considered as quantitatively important in steering environmental food chains and the pandemic character of influenza, with its virus–host disorder–order relationships, reveal that viruses are more than only chemical compounds.^{15,131}

Living cells need as inanimated systems energy inputs to counteract changes induced by information received through a viral genome (section 18.2). Then matter has to be transported from the place of production to the place of demand and consequently ATP has to be equipartioned in the cytoplasm. Energy-driven transport phenomena in cells seem to follow the soliton wave or simply the soliton theory.^{68,148} Soliton-like molecule movements along longer structures¹⁴⁰ could be recently microscopically visualized by observing the transport of kinesine molecules⁵⁹ and mitochondria¹⁸³ in the cytoplasm. Soliton movements occur in one single frequency, at constant speed and without dissipation. If too much energy is involved the wave breaks up into turbulence. If too little energy is given the wave dissipates. A directed matter transport inside of cells, together with the exchange of bits of genetic material either horizontally or vertically by conjugation (plasmid exchange between donor and recipient cells), transformation (uptake of freed DNA by a bacterial cell), and transduction (virus-mediated gene exchange), lets organisms quickly react on perturbations.¹⁹⁵ Newly gained genetic capabilities through viruses, for example, to degrade an unknown substrate, enables cells under changed environmental conditions to convert energy into work and heat ($dQ = C_v \times dT$, where C_v is the heat capacity at a constant volume and dT the temperature change). The thermic energy transport out of a system from a warmer to a cooler site may support the biochemical reactivity of others, but may in the long run lead to an irreversible entropy increase in the known universe. The following sections of this chapter try to unveil strategies against increasing entropy through farming.

18.4 ORDERING PRINCIPLES IN PLANT CELLS

Compartmentation distinguishes eucaryotic microorganisms and plant cells from procaryotic bacteria. The procaryotic endosymbiont, chloroplast, transforms solar into chemical and biological energy (photosynthesis) and the procaryotic endosymbiont, mitochondrion, chemical energy into biological energy. During both processes ATP, a universal molecule that transfers energy between exergonic and endogonic biological reactions, is formed. The compartments chloroplasts and mitochondria in plant cells are rather complex but are reasonably understood.^{150,158}

The chloroplastal evolution started perhaps 3 billion years ago with the engulfment of a phototrophic ancestor bacterium by another organism (endosymbiont theory).¹⁵⁶ Chloroplasts have therefore pigments (chlorophylls), photosynthetic membranes, and many other characteristics with cyanobacteria in common.¹⁵³ They collect with the chlorophylls out of the γ -, X-, ultraviolet, visible, infrared, and radio waves in the sunlight between 400 and 700 nm.¹⁵⁸ The bacteriochlorophylls (BChl) of the free-living phototrophs, which are chemically different, additionally absorb the wavelengths between 715 and 1035 nm. The inhabiting energy of the captured light enables to cleave water into protons, electrons, and O_2 in the oxygenic photosynthesis. Furthermore, an electron transport via various membrane redox systems for generating a membrane potential is initiated. The transported electrons are used as reducing equivalents to bind CO_2 to organic compounds, which are further condensed and reduced to glucose. Glucose comprises a Gibbs-free energy of about 2872 kJ mol^{-1} . Oxygen is then released into the atmosphere (fig. 18.2).

The ancestor cyanobacteria genome of several 1,000 genes was evolutionary reduced to about the 100 genes that chloroplasts have.⁸⁵ Even more reduced to about 10–50 genes is the mitochondrial genome, having *Rickettsia prowazekii* with about 400 genes⁵ and α -proteobacteria *Agrobacterium-Rhizobium* sp. with about 4,000–5,000 genes as ancestors.¹⁷ All withdrawn genes have been overtaken by the plantal nucleus that feeds back on the chloroplastal and mitochondrial transcription of DNA into RNAs and proteins.

Chloroplastal genes initiate the synthesis of chlorophylls and of specific chlorophyll-organizing proteins that force in the light-harvesting complex (LHC) into a specific structure (fig. 18.2).³ Additionally, the chlorophyll-organizing proteins accelerate development.¹⁵⁰ The ordered chlorophyll molecules in the newly created light-harvesting antenna complex (LHAC) efficiently direct the selected sunlight waves into the reaction center (fig. 18.2). Strong excitonic interactions are initiated that concentrate the dipole strength in a few allowed excitonic transitions.¹⁶⁵ Charge separations inside the LHAC elevates the redoxpotential from +0.8 to about 0 volts by simultaneously splitting H_2O into 2 [H] and $0.5 O_2$. ATP, NADH, and heat are produced and become measurable.¹⁵⁸

The several thousand BChls of the free-living phototrophs form, in contrast, an optimal order, evidently by self-aggregation.^{106,165} The self-assembled BChl molecules organize rod-like BChl-aggregates, which are tightly packed in a lipid monolayer-surrounded organelle, called chlorosome.^{150,190} Self-aggregation is considered to differentiate the free-living phototrophic from the chloroplastal system.¹⁸⁸ The BChl-aggregate forming process is increasingly understood and can at least partially be carried out in test tubes. For this purpose zinkchlorins are chemically as long modified as rod-like zinkchlorin-aggregates with very similar spectroscopic properties like the

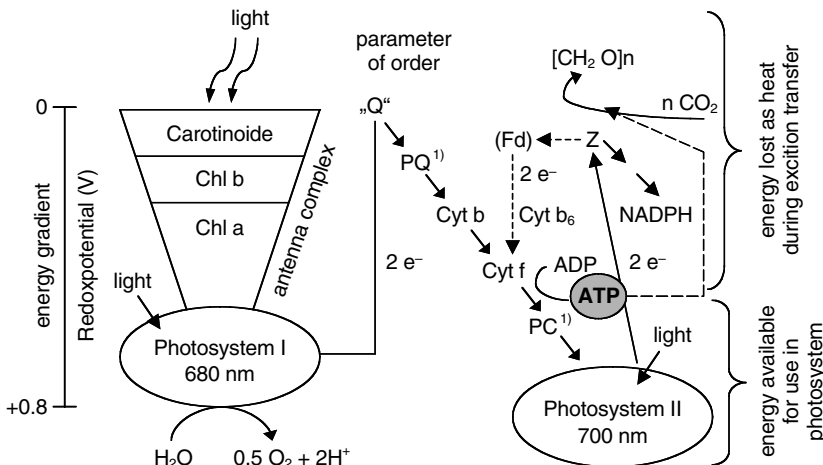


FIGURE 18.2 Photosynthesis, a dissipative and biomass forming process (for details, see the text).

Note: Chl a,b = Chlorophyll; PQ/PC = oxidized/reduced quinone; Fd = ferricyanid; z = unknown

BChls are formed and start to self-organize. These synthetically prepared zinkchlorin-antenna-like molecules, if mixed with a detergent and bacteriochlorins to which fullerenes are attached, collect solar radiation for energizing electrons in the bacteriochlorins. The energized electrons translocated to the fullerenes initiate a photosynthesis-like system. For several nanoseconds an electric charge separation becomes measurable.¹⁵⁰

18.5 MICROBIAL CELL NETWORKS AND CONTROL CIRCUITS

Soil microbes grow in thin waterfilms around solid particles inside of open or clogged soil pores, on plant surfaces, in intercellulars, nodule-like organs, vacuoles, or faunal and grazing animal intestines. Under stress they work together and develop biofilms (chapter 7 in this volume). That working together is necessary for survival was recognized by the English poet John Donne (1572–1631): “No man is an island.” Yet, how in detail this entire process of working together takes place is almost unknown. Billions of bacterial individuals live in soils and many other environments seemingly in harmony (see the introductory section of this chapter; chapters 5–7 in this volume).^{87, 168} But how is the harmony (order) maintained between selfish species in soil pores competing for nutrients or attacking each other (e.g., with deadly antibiotics)?

The numerous microbes living in the various niches of landscapes¹³⁵ take up energy from and give entropy to the surrounding environments in a dynamic and constitutive way. The release of degradation products and heat become measurable, especially in the neighborhood of phase transitions, where the reactivity is highest.^{79, 102, 170, 171} The heat release supports to reorganize the original-like status after a perturbation. Hereby attractors are of importance (section 18.2). Attractors are assumed points in an ideal multidimensional phase space describing, regardless of the starting conditions, the tendency toward which a system will evolve.⁶⁵ Attractors display self-similarity at any scale. They collect positive and negative events on, in, and around the system and work thus against entropy development. The stronger the applied gradient, the greater the effect of the attractor on the system (see the preceding sections in this chapter; fig. 18.1 and fig. 18.2).

Important in regaining order are also the degrees of freedom a system has. The degree of freedom as a physico-chemical concept describes the possibilities a system has to move, or the number of observations (values) in a distribution that vary freely and independently of each other. Atoms freely mobile in space have three possibilities to move, two to move along an axe and one to move on a curve. The freely moveable torso of the prostheca-carrying bacterium fixed to a solid surface (fig. 18.3) should have six degrees of freedom, three of translation and three of rotation.²³ DNA molecules shattered during replication by selective excitations of molecular vibrations¹⁴⁴ have assumably more degrees of freedom than the supercoiled DNA structure.⁶⁸ Since the findings of Henry Taube (Noble Prize, 1983), we realize that even in seemingly stable structures such as supercoiled DNAs the energetic status of the electrons seems rather dynamic and, for example, energy inputs from outside if exceeding a certain threshold can radicalize such seemingly stable systems. Helpful in counteracting radicalization is the availability of organic compounds (e.g., vitamins or pigments) that enable a stable configuration to be regained by easily providing electrons.

Changes in inanimated or living systems are of linear or circular causalities.^{65, 81, 89} Both types of causality have in common a stimulus that initiates a chain of events.

Receptors are activated and nonsynchronized events are degraded. Linear causalities underly the view of connected events by which causal chains are constructed. Implicit in each event is a notion of “agency.” The chain acts to produce an effect, which then becomes the next cause. The weakness lies in the requirements to assign points in a strict order and in time, to the beginning and the end of each chain and to intervening events in the chain. Temporal sequencing is crucial, because no effect can precede or occur simultaneously with its cause. The demonstration of causal invariance must be based on repetition of trials.

Circular causality expands the linear causality concept. Stimuli can arise within the system itself or impinge from outside on the existing state of a system. Top-down macroscopic states

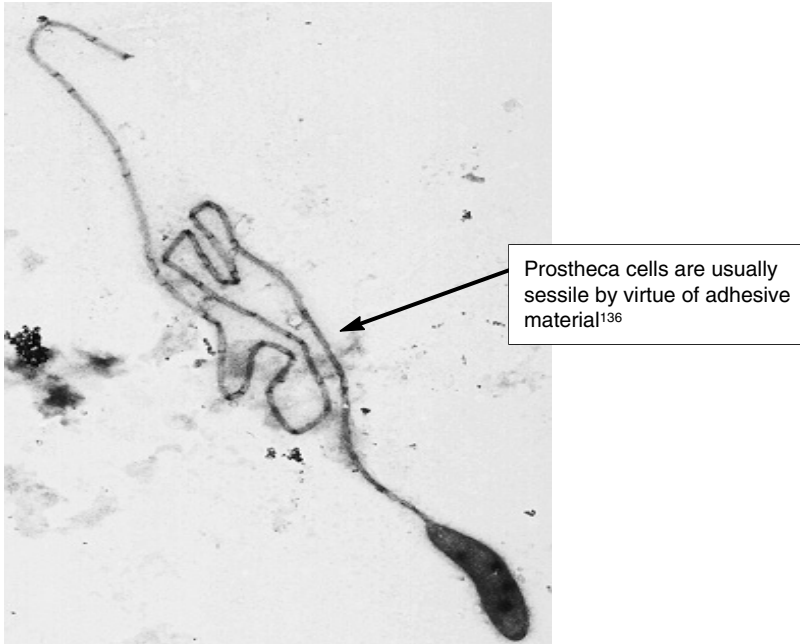


FIGURE 18.3 TEM-picture of an unidentified, prostheca-carrying bacterium found in a nitrifying enrichment culture from an allochthonous Vega soil (experimental station of the Institute of Agronomy, Justus-Liebig University, Giessen, Germany). The TEM-picture was taken by Elke Christ (unpublished, master's thesis, 2001, Institute of Applied Microbiology, Justus-Liebig University). Dr. Martin Hardt, Central Biotechnological Unit, Justus-Liebig University, is thanked for his supports during TEM.

influence microscopic particles that bottom-up and sustain the macroscopic state (sections 18.2–18.4).^{33,79} Events in real time are discrete and marked by changes in the state of the system. A notion of an “agency” does not enter. Multiple scales of time and space are required for reaching different levels of organization. Most stimuli are ineffective, but occasionally one succeeds as a “hit” on the system. A response occurs and becomes a causative factor for future effects, influencing them in a manner particularly subtle, variable, flexible, and of an endless number of possibilities. The concept of circular causalities allows to express more dimensional levels in a hierarchy. Events are intrinsically not reproducible.⁶⁵

In a mixture of microorganisms cultured in a solution to which a signaling metabolite is added in excess and hits the microbial community, or is rigorously excluded, both types of causality are emerging. The result can be a harmonious living together with structures and products, making clear that biofilms are not only accumulations of noninteracting microorganisms but a multicellular organism of increasing genetic diversity (chapter 7 in this volume).^{14,36,95} New resistances or the potential to produce an antimicrobial compound in a synchronized way are developing.^{20,115,203} The interplay between the micro-level (the individual cell) and the macro-level (biofilm) is starting to be extensively studied (chapter 7 in this volume).^{97,121} It is known that extracellular “wetting” fluids allow a collectively coming together of mobile bacteria on hard surfaces,¹²³ that single chemicals (chemotactic signals), chemically composed signals of long-range nature (quorum sensing),^{67,196} or direct cell–cell interactions (e.g., via extracellular polymers [physical signals]) are agents that enable the aggregation of mobile microbes.^{12,29,129} Bacteria grown on nutritionally modified agar plates or in a differently treated culture broth (shaken, unshaken) develop varying cell and colony morphologies (morphotypes). This happens, though the genes carrying nucleotide combinations inside of the DNA-molecules as well as the DNA surrounding protein-modifications (generally histones, nucleosomes) that open at places of high gene transcriptions for allowing the polymerases to function

are highly conservative.^{35,58,157,174,181,194} Genetic information sets and environmental agents steer biofilm differentiation only when signal threshold concentrations are surpassed.^{101,176,196} If a certain signal concentration is reached microorganisms start to assemble, switch stress genes on and off, and develop a new biofilm structure by self-organization (“adaptive cybernetic unit with autocognizance”).^{11,13,125,126} Cell density, growth kinetics, density of metabolic by-products, intensity of food shortage, grazing, environmental conditions (drought, flooding), or genetic exchanges on the gene level (horizontal genomic changes) or on the genome level (vertical genomic changes) determine biofilm formation and structure.^{97,112} The luminescent lightening in the squid *Euprymna scolopes* through the bacterium *Vibrio fischeri*¹⁴¹ and the stress-induced life cycles of *Myxococcus xanthus* and *Dictyostelium discoideum*^{82,160} are elucidating examples.

Mucus components and homoserine lactones are signaling agents in *Vibrio fischeri*, inducing the lux operon coding of the proteins involved in bioluminescence. Only at a certain quorum (number) of *Vibrio fischeri* cells lightening in *Euprymna scolopes* becomes visible. That *Vibrio fischeri* can reach the quorum of bacteria required for bioluminescence *Euprymna scolopes* nourishes it.

The transformation of ubiquitously distributed, motile, and free-living *Myxococcus xanthus* individuals into a fruiting body starts about 4 hours after the onset of nutritional stress. *Myxococcus xanthus* excretes a homoserine lactone (signaling factor A). After a threshold concentration of factor A has been surpassed, about 100,000 *M. xanthus* individuals move together and excrete a second signaling factor C. Factor C is a small protein that stays attached to the cell surface and forces the *M. xanthus* individuals to keep cell contact. A proper cell packing leading to a fruiting body is promoted. Inside of the fruiting body *M. xanthus* cells not reached by signaling factor C differentiate into resistant spores. The spores transported by wind, water, or soil animals to a more favorable environment can germinate again and a new cycle will start.

The social amoeba *D. discoideum* reacts similarly as *M. xanthus* on environmental stress (chapter 10 in this volume; fig. 18.4). Amoebae are a preferred food source for soil-inhabiting nematodes (chapter 19 in this volume). *Caenorhabditis elegans*, for example, approaching *D. discoideum* (chapter 12 and 19 in this volume) then the free-living *D. discoideum* cells release cyclic adenosine monophosphate (cAMP). Pseudopodia are formed, initiated by cAMP and *D. discoideum* individuals creep together to form a fruiting body.⁸²

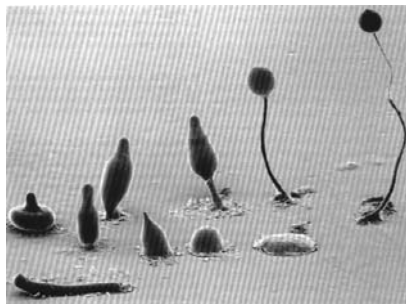


FIGURE 18.4 Life cycle of *Dictyostelium discoideum*.

1. Mound formation of *D. discoideum* after cAMP excretion. The multicellular mound is surrounded by an extracellular matrix, the sheath. Sheath formation protects the amoebae from nematodal feeding.
2. First finger stage formation.
3. The first finger can fall aside and move as a slug with pseudoplasmodia along a light and temperature gradient.
4. Both first finger and slug can differentiate to a mold with fruiting body. Amoebal cells inside of the fruiting body form feeding resistant spores. The sporeforming unit is invaded by nematodal larvae nourished by *D. discoideum*. The grown-up nematodes burst the fruiting body and distribute the amoebal spores.¹⁰¹ One cycle lasts under laboratory conditions about 24 hours. Photo: M.J. Grimson and R.L. Blanton, Electron Microscopy Laboratory, Department of Biological Sciences, Texas Technical University.¹⁶⁰

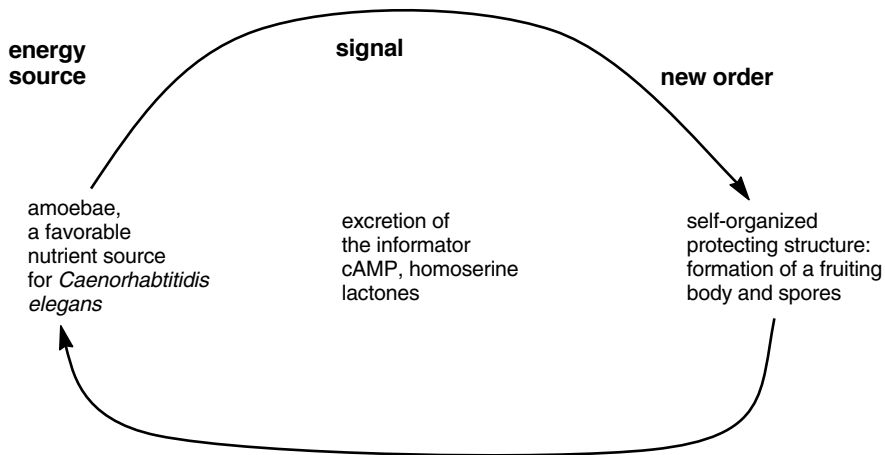


FIGURE 18.5 Feedback mechanism for protecting the amoeba *Dictyostellum discoideum* from being eaten by the nematode *Caenorhabditis elegans* (for details, see sections 18.1–18.5).

The few examples show clearly that signaling (warning) factors as homoserine lactones or cAMP initiate control circuits and a working together starts for counteracting stress (fig. 18.5), but only when a threshold concentration is surpassed. New structures are emerging (fig. 18.4). The changed behavior becomes visualized.

18.6 SYSTEMS OF INCREASING COMPLEXITY AND COMMUNICATION

Farmers approach sustainable productivities by including leguminous plants, bovines and earthworms into their cropping concepts (chapter 1 in this volume). By doing this they try to intertwine the cropping systems more intensively with the worldwide energy cycle. The influxing energy helps to move agricultural production systems far away from a thermodynamic equilibrium and enable them to develop oxic–anoxic interfaces preferentially in biofilms^{38,164} and the neighborhood of roots and symbiont–host relationships.¹⁵⁴ Profiles of soil-inhabiting species, O₂, nitrate, NO₂[−], NO, N₂O, Fe₂O₃, MnO₂, sulfate, CO₂, or fermentation products can be studied on the μm to mm scale by microelectrode-, cryosectioning-, *in situ* hybridization-, and phylogenetic marker-techniques (see preceding chapters in this volume).⁹ The slopes of the above soil-specific profiles integrate diffusion-, consumption-, degradation-, and biomass formation-processes and allow to calculate matter fluxes by $J = -Ds\phi dC/dx$, where J is the flux, D the diffusion coefficient, ϕ the porosity, and C/dx the concentration gradient, respectively (chapter 16 in this volume).^{27,155} The numerous carried-out flux measurements unveiled that it is not easy to mimic *in situ* gradient situations, but ascertained at least that oxygen-tolerant microbes organize the survival of obligate anaerobes in stratified environments. The soil bacterial activities, mostly of a three-dimensional fractal nature, exclude the application of simple one-dimensional models (chapters 16 and 19 in this volume).^{28,192}

18.6.1 SYMBIONT–HOST INTERACTION IN LEGUMES

System functioning is strongly based on communication and cooperation (see the preceding sections in this chapter). An example par excellence for communication and cooperation is the legume-based symbiotic N₂-fixation with its unique organ formed on roots and in a few plants on stems, called nodule (chapter 5 in this volume).¹⁹³

Nodule formation intertwines a saprophytic, N₂-fixing bacteria with the photosynthesis and starts with the release of flavonoids by the plantal partner (fig. 18.6).^{47,122,142,154,180,198} Flavonoids

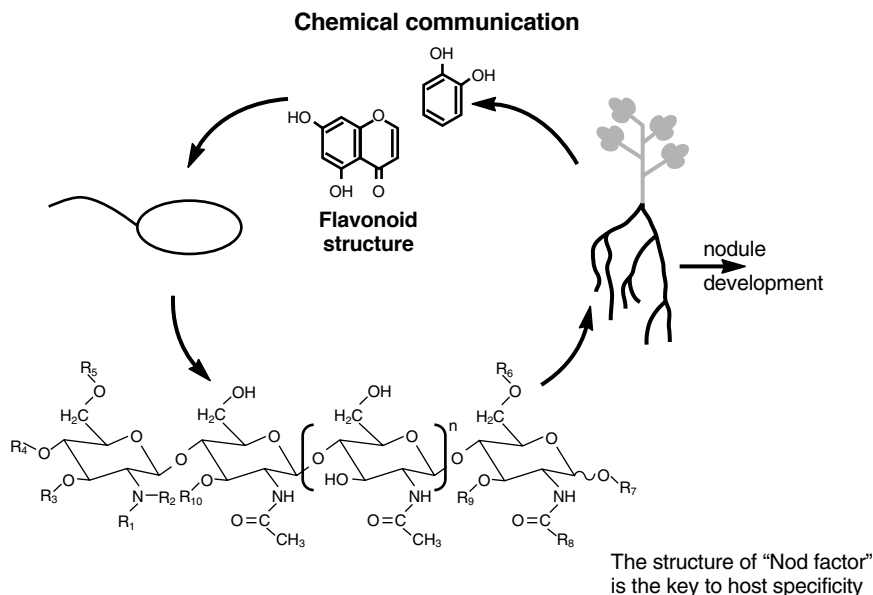


FIGURE 18.6 Feedback mechanism during rhizobium–leguminous host plant communication. A well-organized and sustainable system for millions of years (for details, see sections 18.1–18.6).

signal rhizobial strains to attach to the root surface and to switch on nodulation (*nod*) genes (fig. 18.6). The information for Nod factor production is conserved in many rhizobial *nod* genes (e.g., *nod ABCDEFHLXZ*) and organized in several operons located on the bacterial chromosome or on large (*Sym*) plasmids. Nod factors have a basic lipo-chito-oligosaccharide (LCO) structure. LCOs have the N-acetyl moiety at the nonreducing sugar residue replaced by a fatty acid chain, are common to all rhizobia, but differ widely in their chemistry (fig. 18.6). Nod factors are synthesized in the cell membrane and interact with the Nod signal perception system, a complex Nod signal transduction pathway network that varies from cell to cell type.¹⁸⁰ Nod factors stimulate plantal cells preconditioned by low nitrogen (NH_4^+ , NO_3^-) to divide. Ion fluxes are induced. Influxing Ca^{2+} ions counterbalanced by effluxing Cl^- and K^+ steer tip growth in the root hairs and the cytoskeleton dynamics (actin filaments for directed ATP-transport). Furthermore, influxing Ca^{2+} ions enable plantal cells through exocytosis to deposit new cell wall material and to trigger together with the Nod factors a signal transduction cascade that ends up in the expression of specific genes involved in the symbiotic interaction program. In consequence, root hairs begin to curl at the tips, the cell wall invaginates, and infection threads begin to grow within the root hairs. Differentiated cells of the root cortex are reactivated to divide; the infection thread transverses the outer cell layers and is colonized by multiplying rhizobia. Rhizobia in some legumes reach the root interior by crack entry.

During infection and nodule development many genes (*nodulin* genes) are expressed, which encode cell wall proteins in the root hairs and nodule primordia. *Enod* 2, 5, 10, 11, 12, 40, and *PRP4* genes and root-hair-specific extensin genes encode proline- or glycine-rich proteins (GRPs) and a peroxidase (*ripl*), while rhizobial genes control the cell cycles. Rhizobial genes found their expression in lectins, carbonic anhydrase, chitinase, enzymes of the phenylpropanoid biosynthesis pathway, pathogenesis-related proteins, leghemoglobin, GTP-binding proteins, or protein kinases. The bacterial cell surface components (exopolysaccharides [EPS], lipopolysaccharides [LPS], capsular polysaccharides [CPS]) and plantal lectin, though their roles remain elusive, function during nodule invasion as highly selecting communicators. After having passed this intercommunicating control system, endocytosis at the tip of the infection thread enables the identified and selected rhizobium to invade the mature nodule. At least five different perception systems are becoming

active during nodulation: (1) a generic LCO receptor, (2) a specific Nod signal receptor, (3) a negatively acting perception system for chito-oligosaccharides, (4) LPS-, and (5) Nod-factors.

Endogenous plant signals initiated by auxines, cytokinines, uridine, ethylene (phytohormones), and locally acting nitrate and ammonium regulate location and spacing of the nodules within defined root zones. Auxines inhibit the expression of the early nodulin genes *enod* 2, 12, and 40. Cytokinines induce the genes *enod*12A, a marker gene for inner cortical cell divisions, and *enod*40 encoding strong secondary structures (nodule initiation) and amyloplast deposition. Stele-derived uridine initiates and ethylene downregulates superinfection of the legume roots. Higher concentrated nitrate and ammonium inhibits nodulation completely.

Having invaded the nodule the selected bacteria are enveloped by a plant-derived membrane and are called bacteroids. The membranous envelopment separates the bacterial metabolism from the host cytoplasm and helps by an organized product-waste management system to actively prevent the inhibition of the nitrogenase complex by cytosolic compounds. The repressor NoIR downregulates the Nod factor production and the bacteroid differentiation can start without any disturbance. The bacteroids stimulated by a variety of mostly unidentified signal compounds undergo at least one round of cell division prior to the differentiation into enlarged bacteroids. Host-specific bacteroidal sizes and shapes are induced *in vitro* by microaerophilic succinate metabolism. The nodule nourishes the rhizobial bacteroids with photosynthates and protects it from being eaten. The number of bacteroidal mitochondria and plastids are increasing, but the potential to replicate gets lost. Leghemoglobins regulate the oxygen availability in a way that the nitrogenase complex can function anaerobically by providing simultaneously sufficient O_2 as e^- -acceptors for overall cell metabolism. Through the O_2 -regulating leghemoglobine enough ATP can be supplied to efficiently split the inert $N\equiv N$ into two molecules of ammonia.

The synchronized bacteroidal working together controlled by many genetic and chemical signals covers the bacterial and host N-demand. The dissipation of NH_4^+ into the plantal cytosol is organized in a way that toxic concentrations cannot accumulate and hamper nitrogenase functioning. The complex, signal-induced intercommunication finds its expression in morphological root hair changes and the host-specific nodule- and bacteroidal forms. Senescent and decaying nodules release the bacteroids into a free-living, less-organized stage. A new nodulation- N_2 -fixation cycle can start.

18.6.2 THE RUMEN, A HOMIOTHERMAL, HIGHLY INTERCOMMUNICATING FERMENTER

Less understood than the nodule-rhizobium system is the constantly 39°C-kept bovine rumen with its huge numbers of intercommunicating bacteria, protozoa, and fungi, including their viruses (table 18.1).¹⁶⁴ Table 18.2 groups the about 10^{11} bacterial individuals g^{-1} rumen fluid according to their functions¹⁶⁴ and table 18.3 lists the species of about 10^4 rumen protozoa and about 10^5 fungi g^{-1} rumen fluid as far as they are known.^{46,108} The grass diet is rich in cellulose fibers but low in starch grains, lipid particles, soluble sugars, and proteins. The rumen recycler community provides the bovine with energy (acetate) and proteins and connects the rumen microflora with the photosynthesis.¹⁹⁹

The rumen protozoa (table 18.3) consist of about 16 genera of ciliate protozoa and about 5 genera of flagellate protozoa. The highest diversity of protozoal species comprise the entodiniomorphid ciliate protozoa, with about 100 (*Entodinium*), 7 (*Eodinium*), 37 (*Diplodinium*), 14 (*Eudiplodinium*), 17 (*Eremoplastron*), 30 (*Ostracodinium*), 11 (*Metadinium*), 1 (*Diploplastron*), 1 (*Elytroplastron*), 4 (*Enoploplastron*), 6 (*Polyplastron*), 18 (*Epidinium*), 2 (*Epiplastron*), 1 (*Opisthotrichum*), 8 (*Ophryoscolex*), and 6 (*Caloscolex*) species, respectively.¹⁹⁹ Rumen ciliates, with the tendency to sequester on the rumen epithel, support the bacterial consortium in fiber digestion and are helpful in reducing pathogens. Rumen protozoa digest about 80% of the bacterial biomass, and protect bovines by releasing considerable amounts of NH_3 into the rumen from acidose.¹⁶⁴ Despite the beneficial protozoal contributions, high protozoal numbers are less desired because of the competition for bacterial protein and supported cattle infection caused by *Mycobacterium bovis* protected from *Acanthamoeba castellanii*.^{108,189}

TABLE 18.1
Microbial Populations of Bacterial, Protozoan, and Fungal Fractions Separated from Rumen Fluids by Physical and Chemical Treatments

Microbial population	Mean microbial fraction $\pm$ SE ^a of			
	Whole rumen fluid	Bacteria	Protozoa	Fungi
Bacteria				
Total cell count (10^{11})	36.27 $\pm 11.14^b$	69.54 $\pm 13.49^a$	3.75 $\pm 0.08^c$	0.02 $\pm 0.01^d$
CFU (10^8)	17.32 $\pm 2.18^a$	6.98 $\pm 3.87^b$	ND	ND
Protozoa				
Live cells (10^4)	11.26 $\pm 0.49^a$	ND	3.87 $\pm 1.95^b$	ND
Dead cells (10^5)	35.26 $\pm 9.17^a$	ND	2.42 $\pm 1.21^b$	ND
Fungi				
Total cells (10^5)	4.26 $\pm 1.13^b$	0.17 $\pm 0.02^c$	3.75 $\pm 0.08^b$	13.38 $\pm 0.15^a$
Zoospore TFU ^b (10^4)	9.28 ± 3.66	ND	ND	6.32 ± 1.16

^a ND, not detected. Values in the same row with different lowercase roman superscripts differ significantly ($p < 0.05$).
^b TFU, thallus-forming unit(s).

From Lee et al.¹⁰⁶

TABLE 18.2
The Bacterial Rumen Microflora

Cellolytic:	<i>Fibrobacter succinogenes</i> , <i>Ruminococcus flavefaciens</i> , <i>Ruminococcus albus</i> , <i>Butyrivibrio fibrisolvens</i>
Pectolytic:	<i>Butyrivibrio fibrisolvens</i> , <i>Prevotella ruminocola</i> , <i>Lachnospira mutliparus</i> , <i>Succinivibrio dextrinosolvens</i> , <i>Streptococcus bovis</i> , <i>Prevotella bryantii</i>
Hemicellolytic:	<i>Butyrivibrio fibrisolvens</i> , <i>Prevotella ruminocola</i> , <i>Ruminococcus</i> sp.
Amylolytic:	<i>Fibrobacter succinogenes</i> , <i>Succinomonas amylolytica</i> , <i>Streptococcus bovis</i> , <i>Prevotella ruminocola</i>
Sugar-utilizing:	<i>Prevotella bryantii</i> , <i>Lactobacillus viridans</i> , <i>Streptococcus bovis</i> , <i>Selenomonas ruminatum</i> , <i>Megasphaera elsdenii</i> , <i>Lachnospira multiparus</i> , <i>Succinivibrio dextrinosolvens</i> , <i>Anaerovibrio lipolytica</i>
Lipid-utilizing:	<i>Anaerovibrio lipolytica</i> , <i>Butyrivibrio fibrisolvens</i> , <i>Prevotella bryantii</i> , <i>Eubacterium</i> sp., <i>Micrococcus</i> sp.
Acid-utilizing:	<i>Megasphaera elsdenii</i> , <i>Selenomonas ruminatum</i>
Methane-producing:	<i>Methanobrevibacter ruminantium</i> , <i>Methanobacterium formicium</i> , <i>Methanobacterium mobile</i>
Proteolytic:	<i>Butyrivibrio fibrisolvens</i> , <i>Streptococcus bovis</i> , <i>Prevotella ruminocola</i> , <i>Clostridium aminophilum</i> , <i>Clostridium sticklandii</i>
Ureolytic:	<i>Succinivibrio dextrinosolvens</i> , <i>Selenomonas ruminatum</i> , <i>Prevotella ruminocola</i> , <i>Ruminococcus brevii</i>)
Ammonia-producing:	<i>Prevotella ruminocola</i> , <i>Megasphaera elsdenii</i> , <i>Selenomonas ruminatum</i>

From Russell and Rychlik¹⁶¹

TABLE 18.3
The Protozoal and Fungal Rumen Microflora

Ciliate protozoa	<i>Entodinium</i> sp., <i>Eodinium</i> sp., <i>Diplodinium</i> sp., <i>Eudiplodinium</i> sp., <i>Eremoplastron</i> sp., <i>Ostracodinium</i> sp., <i>Metadinium</i> sp., <i>Diploplastron</i> sp., <i>Elytroplastron</i> sp., <i>Enoploplastron</i> sp., <i>Polyplastron</i> sp., <i>Epidinium</i> sp., <i>Epiplastron</i> sp., <i>Opisthotrichum</i> sp., <i>Ophryoscolex</i> sp., <i>Caloscolex</i> sp.
Flagellate protozoa	<i>Chilomastix</i> sp., <i>Monocercomonas</i> sp., <i>Pentatrichomonas</i> sp., <i>Tetratrichomonas</i> sp., <i>Trichomonas</i> sp.
Fungi	<i>Neocallimastix</i> sp., <i>Piromyces</i> sp., <i>Caecomyces</i> sp.

From Williams and Coleman,¹⁹⁴ Davies et al.⁴⁵

The rumen fungi (table 18.3), majorly represented by the genera *Neocallimastix*, *Piromyces*, and *Caecomyces*, have a high cellulolytic potential.⁴⁶ Rumen fungi forced to live anaerobically have 24–32 hours longer reproduction times. Population densities as high as about 8% of the ruminal microbial biomass can only be reached when ruminants are fed poor-quality forage.¹⁶⁴ The bacterial consortium that excretes bacteriocins keeps the fungal populations low at a high-quality nutrition.⁴⁸ A variety of flagellated cells, previously thought to be flagellated protozoa, have been identified to be zoospores of chytrid fungi (chapter 9 in this volume).

Prehistoric hunters, not knowing that grassy diets allow ruminants to sustain stable microbial communities, started to exploit the photosynthetic potential of grasslands by domesticating bovines. Roughly 100 liters of oxygen reach the rumen per grass diet. The uptaken O₂ supports the degradation of the polymeric lignocellulose structures and is, together with the simultaneously ingested nitrate, rapidly consumed. The sugar pool inside of the homiothermal rumen is increasing. Anaerobes ferment the sugars to acetate, the major energy source of bovines, and less important short-chain compounds like ethanol, butyrate, lactate, propionate, branched-chain volatile fatty acids, and CO₂.²⁶ An average pH of about 6.5 and a redoxpotential between 50 and 400 mV is stabilized. Methane-producing bacteria (table 18.2) remove the surplus H₂ from the system (CO₂ + 4H₂ → CH₄ + 2H₂O) and by doing this regulates the syntrophic fermentation (chapter 16 in this volume). The formed methane is breathed out by the bovines into the atmosphere and contributes to the greenhouse effect with about 16% in maximum (chapter 17 in this volume).⁴⁹ The bacterial rumen consortium has the potential to convert urea, excreted from the bovine bloodstream into the rumen, into protein.¹⁶⁴ The bacterial protein is an important N-source for the bovine.

Breeding programs of the last century increased body weights and milk production from about 2 to 10 liters of milk per day and cellulose-rich diets have increasingly been replaced by starch or protein-rich diets. Ruminants started to suffer under a variety of disorders and increasingly feed additives (ionophores, buffers, antibiotics) were added to the diets.¹⁶⁴ Protein-rich diets partly produced from dead animal material assumingly lead to BSE outbreaks.^{52,53}

The numerous microbial rumen inhabitants and the network of signals behind rumen functioning is beginning to be elucidated (fig. 18.7; see the preceding sections in this chapter).^{62,67,87,128} Diet changes influence the composition of the diverse microbial rumen specialists (table 18.1, table 18.2, table 18.3) and bacteriocins have been identified to regulate the fungal population.¹⁶⁴ Lactobacilli, an important group under the sugar-fermenting rumen bacteria (table 18.1) and evolutionarily adapted to cellulose-rich diets, vary in population density between 2 and 20%. They have continuously supplied with carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins, given up to sporulate.⁸³ Released with the bovine excrements into the soil environment the lactobacilli, together with the rumen microorganisms, are confronted with rough soil conditions, the soil food chain, the autochthonous microflora, O₂, NO₃⁻, NO₂⁻, growth inhibitors, frequently changing substrate availabilities, sunlight, sedimentation, or salinity (see the preceding chapters and chapter 19 in this volume). Earthworms feed preferently on bovine excrements of ruminants, which are predominantly enriched with gram negative to oxygen-free conditions adapted bacteria (chapter 14 in this volume).¹¹⁶

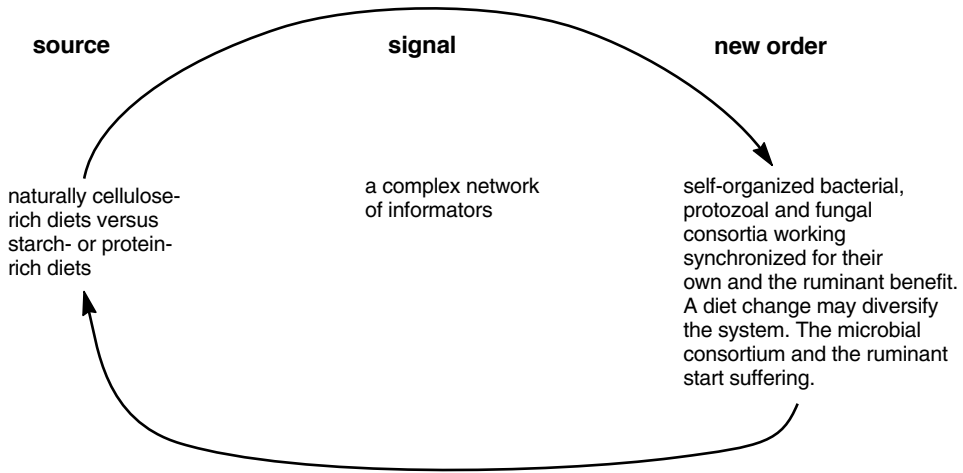


FIGURE 18.7 Microbial feedback mechanism for the nutritional and health benefit of bovines (for details, see sections 18.1–18.6).

Earthworm guts may provide for those bacteria a niche to multiply and to adapt, if not digested, for survival after being distributed in soils with earthworm excrements.

18.6.3 THE EARTHWORM INTESTINOSPHERE, AN AGRICULTURALLY IMPORTANT FLOW-THROUGH SYSTEM

Earthworms are mediators between the inanimated and animated soil world because they ingest animal excrements, plant debris, and mineral soil components.¹¹⁶ They shred the ingested material to increase the surface area. The own enzymes and the simultaneously taken-up bacteria, fungi, protozoa, nematodes, and tardigrada (chapters 4–13 in this volume) can now mineralize the engulfed soil material more efficiently. The nutrients released during digestion benefit the earthworm (chapter 14), the earthworm inhabiting microflora, and the environment in which the earthworm releases its excrement (fig. 18.8; chapter 19 in this volume).^{24,51}

On average earthworms consume about 120 kg carbon and about 10 kg nitrogen $\text{ha}^{-1} \text{yr}^{-1}$. Each ingested gram contains about 5×10^8 colony-forming units (cfu) of bacteria, which assimilate about 9–19% of the ingested carbon during the gut transit.⁵¹ Actinomyces contribute about 2.6×10^7 cfu. The most probable numbers (MPN) of the aerobic, fermenting, ammonium, and nitrite-oxidizing, denitrifying, nitrate-dissimilating, and methanogenic bacteria increase during the gut passage in comparison with bulk soils from 9×10^6 to 3×10^8 , 2×10^5 to 1×10^7 , 2×10^4 to 2×10^5 , 2×10^4 to 3×10^5 , 2.1×10^4 to 6×10^6 , 4×10^5 to 1×10^7 , and 2×10^1 to 1×10^2 , respectively. Genome analysis (16S rDNA sequencing pattern) of the gut bacteria reveal close relationships with *Sinorhizobium* sp., *Ralstonia picketti*, *Ralstonia brassicacearum*, *Aeromonas hydrophilia*, *Aeromonas media*, *Buttiauxella warmboldiae*, *Enterobacter asburiae*, *Flavobacterium johnsoniae*, *Clostridium glycolicum*, *Clostridium putrefaciens*, *Clostridium manganotii*, *Bacillus mycoides*, *Paenibacillus* sp., *Paenibacillus burgondia*, *Paenibacillus borealis*, and *Paenibacillus amylolyticus*.^{60,88,92}

The information about the protozoa in earthworm guts and excrements is very limited compared to the gut bacteria (chapters 11 and 14 in this volume).²⁴ It is reported that more than 180 monocystid gregarines live or parasitize on earthworm tissues, but nothing is known about the role protozoa play in the earthworm gut and how they are surviving. Distinct groups of astomatous ciliates seem to resist digestion in insects by seemingly losing the capability to encyst.⁹⁴ For the protozoa residing in the earthworm intestinosphere this has to be confirmed.

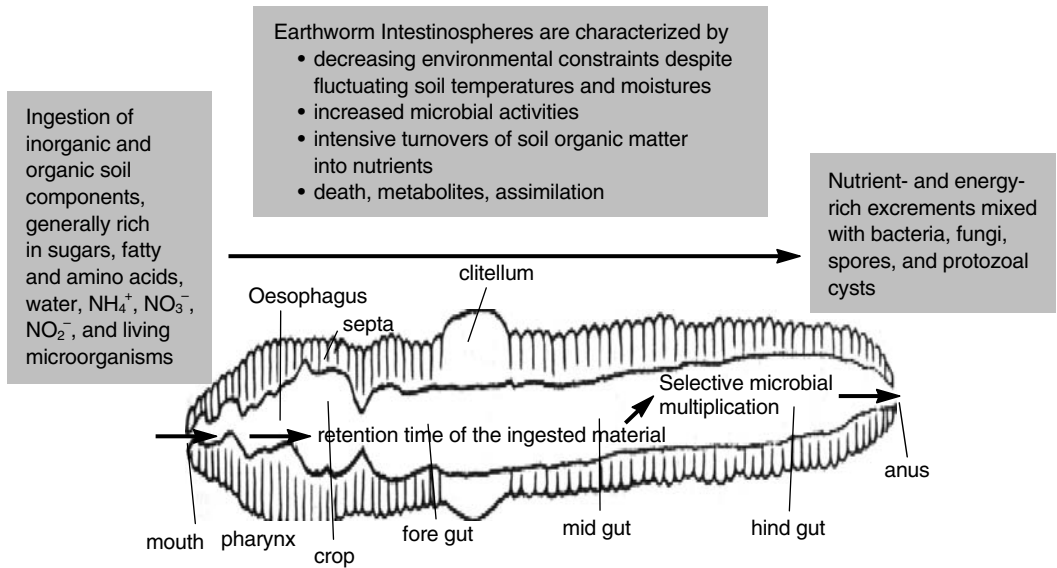


FIGURE 18.8 Schematic longitudinal section and external morphological characteristics of a poikilothermal lumbricid earthworm (adapted from Brown²⁴)

The available information about the fungi entering the earthworm gut is similarly poor. Food preference tests with ecological-relevant earthworm groups and nine fungal species—*Cladosporium cladosporioides*, *Rhizoctonia solani*, *Mucor* sp., *Trichoderma viride*, *Fusarium nivale*, *Phlebia radiata*, *Glaeophyllum trabeum*, *Coniophora puteana*, and *Coriolus versicolor*—revealed that *F. nivale* and *C. cladosporioides* are preferentially eaten by earthworms.²¹ Next in the food preference are the fast-growing species *Mucor* sp. and *R. solani*. Successional fungal species seem not only to serve earthworms as a food source but interestingly serve also as a signal for detecting fresh soil organic resources. Basidiomycetes as a food source are refused.

Total carbon and nitrogen are about 2 to 4, 4 to 5, and 5 to 8 times, respectively, higher in the earthworm gut water than in the soil environment.^{88,92} The amino acid content of gut is approximately 40-fold greater. Sugars like glucose and maltose, in soils not detectable, enrich in the earthworm guts and reach concentrations of about 32 and 3 mM, respectively, although acetate, formate, lactate, and succinate are low-concentrated. Only nitrate is higher concentrated in the soil solution. Evidently the oxygen-free, pH-neutral aqueous gut phase of earthworms provides favorable conditions for anaerobic respiration (denitrification) and fermentation.^{88,92}

Food quality and temperature regime regulate the poikilothermal gut and the transit time in the microbial activity in earthworm intestines.²³ Gut transit differs in earthworm species considerably and ranges between 1 and 20 h.²³ Earthworms with short gut transit times are *Millsonia anomala* (1–2 hours), *Pontoscolex corethrurus* (2–4 h), *Aporrectodea rosea* (1–2.5 h), *Eisenia fetida* (2.5 h), *Aporrectodea caliginosa* (1 h), and *Octolasion lacteum* (2 h). Significantly longer gut retention times occur in *Lumbricus rubellus* (6–8 h), *Lumbricus festivus* (9–15 h), and *Lumbricus terrestris* (8–20 h).²⁴ Short gut transits <1–2 h assumingly limit reproduction and survival of the ingested microorganisms, but surprisingly though not well understood, microbial cell numbers are also increased in earthworm excrements with gut transits >2 h.^{24,25} Microbial strategies to resist and survive earthworm digestion are spore formation, encystment, the potential to grow anaerobically and syntrophically, the capability to use a broad spectrum of electron acceptors, and fast reproduction under high and low substrate concentrations (fig. 18.8; chapter 16 in this volume). Epigeic, endogeic, and anoeic earthworms, hooked into the overall energy flow, move along water and temperature gradients through soils or are guided by their genetically predetermined nutritional

behavior. During their movements they deposit the nondigestable residues enriched with an anabolizing and catabolizing microflora spotwise on and in soils. With excrements the digged tubes are also refilled.^{9,24} Earthworm excrements improve soil texture and structure and are highly fertile. Plants are penetrating the refilled earthworm tubes and are benefitting from the nutrients. Farming reduces the earthworm abundances by destroying the channels and the earthworms themselves (chapters 1 and 3 in this volume).^{116,169} Farming effects not only earthworms but impairs also the nest-constructing, state-organizing ants, another agriculturally important soil animal group.

18.7 STATE-ORGANIZING ANTS AND THEIR WORKING-TOGETHER PRINCIPLES

Ants grow fungi since the early Tertiary and predate human agriculture by about 50–60 million years.¹³⁴ They are skilled biotechnologists and ecosystem engineers and assumingly turn more soil around during nest construction than earthworms do.¹⁹⁷ *Lasius neoniger*, often found in arable land, turn around the upper 30 centimeters of a hectar cornfield once every 2,800 years.^{197,45} For nest construction ants must have the following skills.¹⁴⁹ At a minimum, an antal colony must find a suitable nest site and safely bring its entire population there. Typically, it must evaluate several potential sites, compare them, and choose the best one. Finally, the colony must reach consensus on a single site, rather than splitting its members among several. Ants seem to achieve all these ends without any form of central control. Accordingly this chapter section will deal with ant state functioning.

18.7.1 ANTS IN AN AGRICULTURAL LANDSCAPE: IMPORTANCE AND DISTRIBUTION

Ants belong to the order Hymenoptera (family: Formicidae). Worldwide there are about 9,500 species known, but only a few of them have the capability to survive the vagaries of climate and frequent agricultural perturbations.^{4,41,44,111} Compared with the known ant species is the antal diversity in the agricultural land use mosaic of the Lahn-Dill Bergland, consisting of arable (A), fallow (F), and grassland fields (G) reduced to about 0.3% or a total of 27 species (table 18.4).^{41,44} The specific climatic conditions, the land morphology, and the land use are mainly responsible for the varietal reduction. The antal species found in the Lahn-Bergland mosaic are listed in table 18.4. Migrators from forest edges into the open landscape are the typical forest species *M. ruginodis*, *M. lobicornis*, *L. acervorum*, *F. fusca*, and the cursorial active formica species *F. rufibarbis*, *F. cunicularia*, *F. pratensis*, *F. polycatena*, *F. rufa*, *F. fusca*, *F. rufibarbis*, *F. cunicularia*, *F. pratensis*, *F. polycatena*, *F. rufa*, *F. sanguinea*, *M. ruginodis*, *M. lobicornis*, *Lasius platythorax*, and *L. acervorum*. Species of peat land and humid meadows such as *M. vandeli* prefer to settle in fallow plots, while the more thermophylic ants *M. sabuleti*, *M. schencki*, *M. rugulosa*, *Tetramorium caespitum*, *Tapinoma erraticum*, and *L. alienus* prefer the grassland. Highly resistant against anthropogenic disturbances are the vagabonding ants *M. scabrinoides*, *M. rubra*, and *M. ruginodis*, and various *Lasius* species. *L. flavus* and *L. niger* dominate the centers of agricultural field sites by depending as social parasites on the availability of hosts, generally other insects.

Ant species found in 20 hand-sampled, randomly chosen 2 m² plots of the Lahn-Dill Bergland amounted to 0–4 (A) or 3–7 (G, F), respectively. The nest densities per 100 m² ranged between 0 to 60 (A), 40 to 180 (G), and 80 to 200 (F), respectively.⁴¹ During construction activities and brood hatching, ants inside and around the nests deposit considerable amounts of material rich in carbon, nitrogen, and phosphorous. The stored material and the released nutrients improve biopore formation (water infiltration), soil fertility, seed germination, and plant growth.^{19,32,66} Accordingly, are antal mounds often densely penetrated by roots.¹¹¹ This crop-improving, beneficial antal effect may be relativated by the fact that *Lasius* species may attack surface-dwelling, plant growth-promoting arthropods.^{43,50,54}

TABLE 18.4
Ant Species Found in the Agricultural Land Use Mosaic of the Lahn-Dill Bergland Consists of Arable (A), Fallow (F), and Grassland Fields (G)

Camponotus species	<i>Camponotus ligniperda</i> Latr. 1802 (A, G)
Formica species	<i>Formica cunicularia</i> Latr. 1798 (A, F, G), <i>Formica fusca</i> L. 1758 (A, F, G), <i>Formica polyctena</i> Förster 1850 (F), <i>Formica pratensis</i> Retzius 1783 (A, F, G), <i>Formica rufa</i> L. 1761 (F), <i>Formica rufibarbis</i> Fabr. 1793 (A, F, G), <i>Formica sanguinea</i> Latr. 1798 (F, G)
Lasius species	<i>Lasius alienus</i> Förster 1850 (F, G), <i>Lasius distinguendus</i> Emery 1916 (A, F), <i>Lasius flavus</i> Fabr. 1781 (A, F, G), <i>Lasius fuliginosus</i> Latr. 1798 (F), <i>Lasius jensi</i> Seifert 1982 (A, F), <i>Lasius meridionalis</i> Bondroit 1919 (A, F), <i>Lasius mixtus</i> Nyl. 1846 (G, F), <i>Lasius niger</i> L. 1758 (A, F, G), <i>Lasius platythorax</i> Seifert 1991 (F), <i>Lasius umbratus</i> Nyl. 1846 (A), <i>Lasius sabularum</i> Bondroit 1918 (A)
Leptothorax species	<i>Leptothorax acervorum</i> Fabr. 1793 (A, F, G)
Myrmecina species	<i>Myrmecina graminicola</i> Latr. 1802 (G)
Myrmica species	<i>Myrmica lobicornis</i> Nyl. 1846 (A, F, G), <i>Myrmica lonae</i> Finzi 1926 (G), <i>Myrmica microrubra</i> Seifert 1993 (A, G), <i>Myrmica rubra</i> L. 1758 (A, F, G), <i>Myrmica ruginodis</i> Nyl. 1846 (A, F, G), <i>Myrmica rugulosa</i> Nyl. 1846 (A, F, G), <i>Myrmica sabuleti</i> Meinert 1860 (A, F, G), <i>Myrmica scabrinoides</i> Nyl. 1846 (A, F, G), <i>Myrmica schencki</i> Viereck 1903 (A, F, G), <i>Myrmica vandeli</i> Bondroit 1920 (F)
Ponera species	<i>Ponera coarctata</i> Latr. 1802 (A)
Stenamma species	<i>Stenamma debile</i> Förster 1850 (F),
Symbiomyrma species	<i>Symbiomyrma karavalevi</i> Arnoldi 1930 (A)
Tapinoma species	<i>Tapinoma erraticum</i> Latr. 1798 (A, F, G)
Tetramorium species	<i>Tetramorium caespitum</i> L. 1758 (A, F, G).

From Dauber and Wolters,⁴² Dauber⁴¹

18.7.2 GENETIC AND BEHAVIORAL DIVERSITY

Ants live in colonies.¹¹¹ The size of individual colonies ranges from a few dozen to half a million or more. All individuals are predetermined into winged fertile females (queens), wingless infertile females (workers, soldiers), wingless males (soldiers in some few ant families), and winged males, whose only task is reproduction. Female ants develop from fertilized eggs and males from unfertilized ones. Females become queens or workers (soldiers) depending on the type of nutrition they receive. Queens bite or scrape off their wings after having excavated a chamber and proceed to lay eggs for the rest of their life (up to 15 years). Workers, which guard mother queens when they move from one nest to another, minimize the danger for the queen at any time and can be the majority of colony members in some species.⁶⁴ Other pattern variations during new colony formation based on genetic information are that in some species the queen cannot establish a colony herself and is adopted by workers of another colony,¹⁹² or slave-making ants raid nests of other ant species and carry off larvae or pupae to serve as workers,¹⁶⁷ or fighter males of the ant *Cardiocondyla* kill each other but tolerate their winged rivals.⁵ The latter behavior is called “social parasitism.”

Ants are carnivorous, herbivorous, or omnivorous. *L. niger* and *L. flavus*, dominating the centers of agricultural soils, eat preferently honeydew from plants infested with aphids (chapter 8 in this volume).^{19,61} Harvester ants store seeds, stimulate their germination, and feed on them, while others grow fungi inside of their nest for feeding purposes.^{40,4,179} Fungi-growing ants nourish large colonies with about 10³–10⁶ workers and can become real agricultural pests.⁸⁴ Vertical fungal cultivar transmissions between antal generations have lead to a long-term ant–fungus coevolution. The coevolved fungal cultivars are dispersed from the parent to the offspring nests by the queens.⁷⁶

18.7.3 NEST CONSTRUCTION AND PATTERN FORMATION

L. niger species adapted to agricultural perturbations and ant species living in less disturbed areas excavate earth and construct subterranean nests for feeding their broods. Some ant species construct nests with one dome; others such as *Leptothorax longispinosus* construct polydomy nests with specific tunnels and galleries.¹⁸² Antal nest constructions have longevities between several months and about 10 years. In agricultural soils ants have to industriously reconstruct their nests after each disturbance. Antal nest construction provides a classic example for biological pattern formation.

Nest construction at the individual (worker) level and sophisticated outcomes at the colony level follows specific behavioral rules. None of the workers has a global knowledge of the final structure but by working together they create fairly sophisticated nests. Nest extension or reduction is regulated by the colony size and documented by the fact that in late autumn, as winter approaches, ants tend to rebuild their nests so that they are smaller than at the height of summer.^{4,64,152}

Nest construction starts with an intensive turning around of soil particles and the transportation to the nest location. Workers that remain within the nest organize the relocation of building blocks. The following principles are behind progressive order during nest building.^{63,152}

1. The cluster of adult ants and their brood is a template for the nest that tends to end up in circular form.
2. Already used stones feed positive back on the deposition of stones newly brought in (snowballing effect).
3. Local accumulations of stones result in a negative feedback, because one another's removal is inhibited.
4. Workers (foragers) not directly engaged in building activities influence construction through collisions.
5. The maximal digging rate during nest enlargement is closely proportional to the group size.

The maximum digging frequency α is modulated in relation to the volume plateau (V_s). Each individual cast member and finally the whole building activities of a group follow the information given by V_s . Other signals that steer nest patterns are of a chemical nature or derive from body contacts. The higher the group size, the higher the chemical concentration and the more individuals are stimulated to dig. With forthcoming nest enlargement, the signal concentrations and the stimulation to dig decrease.¹⁵²

18.7.4 CASTE DIFFERENTIATION AND DIVISION OF LABOR

The hallmarks of insect societies and the ecological success of ants are caste differentiation and division of labor.³¹ Reasonably studied in this respect are fungi-growing ants, because they are an agricultural pest.

Fungi-growing ants are substructured into foragers and waste managers.³⁹ Forager ants collect vegetable matter to grow a symbiotic basidiomycete of the family *Lepiotaceae*. The symbiotic basidiomycete, not able to digest cellulose,¹ resides in underground chambers and represents the sole food source of the developing brood. The adult foragers, in contrast, obtain more than 90% of their energy requirement from the plant sap that they imbibe during cutting and incorporating the harvested leaf fragments into the fungus garden.¹⁶¹ Thus, foraging ants and the fungus have conflicting requirements for the quality of the plant material to be harvested. Ant workers may prefer resources that support maximal growth rates of the fungus, more or less irrespective of the attractiveness of the plant sap being imbibed during the harvesting process, or may base their decisions about the quality of a given resource on the immediate availability of energy to support their foraging activity. This decision making is important for maximizing fungal yields and to sustain high colony productivity.

A third decision-maker is a virulent parasitic fungus of the genus *Escovopsis* (Ascomycotina), which infects the basidiomycetal gardens. Ant workers, responsible for fungi growing, keep the numbers of *Escovopsis* sp. low by carrying upon the cuticle region a filamentous bacterium of the genus *Streptomyces* sp. that produces antibiotics specifically targeted to suppress the growth of *Escovopsis* sp.⁴⁰ Another measure to avoid *Escovopsis* infections is that worker ants are trained as waste managers. Waste-managing ants are subdivided into a transporter and heap worker caste, and into further partitions within the nest. Formidable quantities of wastes consisting of old fungus gardens, culture medium, and dead workers have to be cooperatively deposited in special underground chambers or in external dumps and heaps. Waste management accounts for approximately 10% of work performed outside the nest. The moving inside and outside of the nest is organized in a way that both foraging and waste-managing workers have no contact with each other. Closely related, sympatric species unveil different solutions to waste removal. Explaining why it is hypothesized that interspecific differences in disease susceptibility, particularly to *Escovopsis* sp., could be the reason.

18.7.5 MOBILITY AND ORDER

Antal genetic and behavioral diversities result in an individual colony odor,⁹⁸ whereas mobility is important to perform a range of vital activities such as feeding, dispersal, mating, nest building, foraging, waste management, or defense. Mobility is fundamental to coordinate and maintain a highly structured and organized society.^{5,84,192}

Ants are poikilothermal, as are all other insects and earthworms. Their seasonal mobility enables a widespread dissemination of information, correct task allocation, and dynamic interactions.^{117,118,134} Without any external stimulus single isolated worker ants exhibit spontaneous motor activity. A low-dimensional disorder displays. Once the ants are allowed to interact in a colony, this disordered behavior tends to vanish. Disordered movement turns into physiological rhythm driven solely by the interacting ants. The suddenly emerging physiological rhythm translated into mobility is generated by networks of coupled cells and is termed “central pattern generator.”¹³⁰ Ants switch only to move when they sense that a quorum of nestmates is present. A specific type of quorum sensing is the recruitment of nestmates.¹⁴⁹ The phenomena of periodic mobility in ant colonies can be summarized as follows¹³⁰:

1. Single individuals are chaotic in their spontaneous activation.
2. Interactions among the objects lead to an activation and more order. This process is clearly synergetic.
3. The transition from disorder into periodic cycles on a colony level is a function of density (diffusion phenomena, section 18.3).
4. Increasing numbers of ant individuals added to a lattice and evaluated in their nest-building behavior by using a scale from 0 to 1 start to self-organize at a density value of around 0.2. At this value the maximum of disorder (entropy) is reached (point of transition from disorder to order).

Ant scouts, successful in finding a food resource, return with this information to the nest and perform a motor display for recruiting nestmates. This means running, body vibration, interindividual body contact, self-grooming, food transmissions, and sound production.⁷⁵ An oriented movement directed by mechanical stimuli, in particular the tendency to follow a wall or another mechanical border, is called “thigmotaxis.” Thigmotaxis helps nest ants to find a way, but the shortest way, in returning from large-scale foraging excursions by possessing an odometer, which allows them to organize mobility by path integration.^{37,119,201} Task partitioning, where the main task (food collection) is splitted into subtasks by passing the material through bucket brigades from one worker

to another, is an additional possibility ants have available to organize directed mobility during food transport back to the nest.⁶

Directed mobility through chemical signals is another main currency of communication during foraging. Pheromones, not-identified substances excreted from a Dufour's or poison gland,⁹⁸ and/or enhanced amounts of CO₂,^{40,75,105} mainly deriving from the cultivated fungi, steer the mobility behavior of ants.¹⁶¹ The signal that guides ant mobility toward honeydew-producing aphids is the quantitative difference in excreted phloem sap.⁶¹ Only high honeydew-secreting aphid populations that can send out a quorum of ant mobility-directing chemicals are selected by a mutualistic *Lasius niger* partner. *Lasius niger*, in return, provides the aphids protection.⁶¹ The quorum of cuticular hydrocarbon profiles as an informational cue for constraining an optimal sex allocation in ants (optimal rearing ratios of females and males) determine, together with the actual temperature as a regulator for attenuation and degradation of the signaling chemicals, indirectly the mobility in a poikilothermal ant colony.^{22,162}

Each signal ends up in the antal nervous system that is interconnected by a network of ganglia located in specific regions along the body and directly connected to the brain. The brain, the largest aggregation of neural tissue, is directly wired to the eyes and the sense organs in the antennae, the places of complex information processing and control tasks.^{77,96}

18.7.6 CHANGING TASKS AND NEURONAL MODIFICATIONS

Alterations in antal caste-, sex-, and working-class ratios as well as the foraging and living together patterns are controlled on the colony level. Behavioral transitions coincide frequently with structural and functional changes in the brain (fig. 18.9). Hormones as documented in birds, honeybees, and ants are seemingly important in controlling neuronal modifications and ultimately the brain.^{69,96}

Particularly reproductive females (queens) have to go through strong behavioral transitions. Briefly, young winged females in their dark subterranean nests are cared for by workers until the proper time for their mating flight arrives. After copulation the males are dying. A completely new female life phase starts. The mated females excavate a subterranean nest and produce generally from their metabolic reserves worker eggs. The first offsprings, small workers, are raised in the dark nest. During their mating flight the young females depend on visual and chemical cues that they have never experienced before. After the mating flight a completely other behavioral program starts: nest excavation and egg-laying in the dark. Immediately, the phototactic switches into thigmotaction. The releasers for these behavioral changes are not really understood but it is for sure that light conditions are not the cause.⁹⁶ The whole visual system in queen ants is downregulated, and the optic nerve, cell body diameter, and medulla volume are reduced. This is probably due to the fact that photoreceptors are metabolically expensive, costing roughly 10% of the total energy.¹⁰⁵ The downregulation of brain functions could be shown in *Harpegnathos saltator*, *Mesopergandeia*, and *Pogonomyrmex rugosus*.^{77,96} In "normal" ants, to which odor is more important than vision (e.g., carpenter ants *Camponotus* spp.), the volumetric ratios of the visual brain centers (medulla and lobula) and the antennal lobes (brain centers concerned with odor processing) ranges between 0.43 and 0.5. In the visual predators *Myrmecia* spp. and in the visually foraging desert ant *Cataglyphis* spp., the above ratio is 0.71 and 1.06, respectively. *Harpegnathos* workers and the extremely visual ant species *Gigantiops destructor* surpass all other ants with ratios of 3.66 and 5.03, respectively. The brain size of a non-egg-laying *Harpegnathos saltator* worker compared with that of an egg-laying worker or gamergate figure 18.9. The behavioral and physiological transition from a nonlaying worker to a gamergate happens quite often in *Harpegnathos saltator* species because many workers are inseminated by males and replace the queen, if necessary. During this job change the brain volume decreases substantially by about 26%. Predominantly reduced in the gamergate brain are the sections medulla, lobula, antennal lobes, mushroom body, and central body.

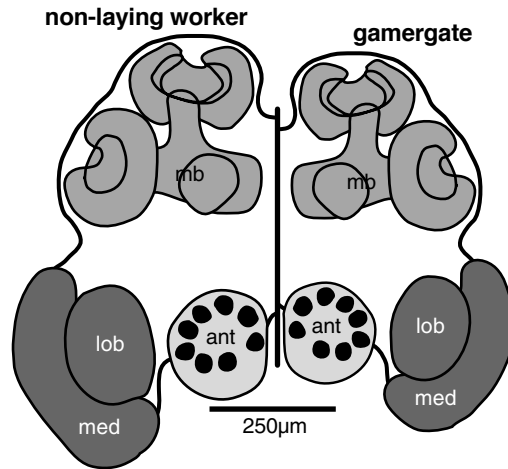


FIGURE 18.9 Schematized drawing (frontal view of the brains of a non-egg-laying worker (left) and an egg-laying worker (gamergate, right) of *Harpegnatos saltator* showing the large visual centers (*med*, medulla; *lob* lobula), the olfactory centers (*ant*, antennal lobe), and the mushroom bodies *mb* (central body omitted); dorsal is top.⁷⁷

Interestingly, less reduced are the optical lobes (only 20%).⁷⁷ Brain size changes correlate with the behavioral patterns and seem to be an universal phenomenon in ants.^{77,96}

18.7.7 LIVING TOGETHER IN SELF-ORGANIZED PATTERNS

As outlined ants have evolutionarily developed a variety of behavioral, physiological, or polymorphic transitions, resulting in a task-partitioned living together, an improved individual and colony fitness, and a high plasticity (see the preceding sections in this chapter).^{34,117,118,119} During their ontogenesis, inside arable land-residing *L. niger* colonies, a progressive integration of individual foragers develop a network of chemical and tactile communications. An integrative defense system is developed that provides the foraging system with an excellent model how single colony members with limited skills are integrated into a collective foraging and defense behavior. The individual level contributes with decisions on the motivational state of foraging workers, on size, location, and quality of the leaf fragment to be cut, and on how to communicate for nestmate recruitment. The collective level, first, provides the quantitative information to assess the proportion of trail-laying *L. niger* ants or food volume and to regulate defense.¹¹⁸ For decision making and processing in nest defense by *L. niger* collectives and in finding foraging areas (e.g., honey-dew-producing aphids at plant roots), three general information systems must be available¹⁶⁵:

1. A recruiting system that allocates more workers to more valuable resources.
2. The simple decision rule of individual ants to become more aggressive in response to increased numbers of nestmates nearby.
3. The gradual change in the proportion of fighting and fleeing ants.

Individual ants are incapable of collecting and processing enough information to assess a situation independently. Only antal networks termed social context or collective intelligence are able to act in a successful way.¹¹⁸ A collective intelligence comparable to antal networks seem already to exist on the procaryotic level in biofilms (section 18.5; chapter 7 in this volume). Social insect organization, as recently discovered in polymorphic worker casts of *Acromyrmex echinator* with small workers specializing in intranidal tasks and large workers carrying out foraging, is

controlled by genetic and environmental cues.⁹⁰ Genetic cues have to reach similarly as chemical or thigmotactic signals the necessary concentrations to initiate brain reduction in *Acromyrmex echinator* or to carry out self-organized foraging. Both are first possible on the colony level. Expected to be involved in self-organized foraging are regulatory positive and negative feedback loops. Suddenly, collective antal responses, far from being understood, are emerging.¹⁶¹ The agricultural point of view is of interest and is a major challenge to understand how leaf-cutting ants, which can reduce crop yields significantly, perceive on colony-level changes in fungal productivity. Informator to alter the own quality criteria for plant selection seems to be carbon dioxide. Crop protection will improve when plant selection is reasonably understood.

18.8 ENTROPY REDUCTION ON AGROECOSYSTEM SCALE

Agricultural production systems can approach the productivity of natural successions with numerous coevolved plants, animals, and microorganisms (table 18.5). This indicates that even pervasive perturbations through farming do not prevent high biomass production. A cropping system with minor perturbations through farming is the traditional shifting cultivation practiced in West Africa (chapter 1 in this volume).² Shifting cultivation means that areas of soil are cleared of the steady-state vegetation and cultivated for a few years during which the fertility declines because of the loss of organic matter and mineral plant nutrients. When yields become too small the areas are allowed to revert to the natural vegetation and fertility gradually restores. The extent of the perturbation decides about the time needed for recuperation. When the soil was untilled and crops were planted in holes made with “dibbling sticks,” the soil recuperated in about 10 years. If the soil was tilled, that is, turned over with hoes, the self-organized recuperation tooks seemingly about 50 years.²

Large-sized agricultural fields of 10–50 ha and more are increasingly established worldwide because of labor and machinery costs. Large-sized field plots comprise various soil types, slopes, and valleys (chapter 1 in this volume). Soil and nutrient homogeneity is achieved with diverse soil tillage techniques and crop-adapted fertilizer application strategies (e.g., GPS-guided fertilization). Soil homogenization and pesticide applications, parts of the agricultural management, cause biodiversity shifts in and on the soils as well as loss of species (chapters 1, 5–14 in this volume; section 18.7.1.). Despite the above farming impacts, high crop productivities can be preserved over years

TABLE 18.5
Netto Primary Productivity in g Biomass Dry Weight per m² and Year of Natural and Agriculturally Used Areas in Different Countries of the World

Country	Agricultural productivity g m ² yr ⁻¹	Natural productivity g m ² yr ⁻¹	Ratio agricultural/natural productivity
Democratic Republic of the Congo	180	1,960	0.10
Kenya	350	1,300	0.13
Nigeria	150	890	0.17
Cambodia	310	1,800	0.17
Bolivia	280	1,500	0.19
Brazil	310	1,620	0.19
Spain	510	750	0.68
Germany	1,130	1,190	0.95
Belgium	1,290	1,210	1.07
The Netherlands			
Luxembourg			

From Esser⁵⁶

(table 18.5), revealing that the soil microflora and fauna seems to adapt to the new situation. Species introduced airborne, by fertilization or faunal droppings, substitute other species. Additionally, species can migrate into agricultural sites from nearby forests, pastures, and fallow lands (chapter 4 in this volume; section 18.6 and section 18.7). Large-sized agricultural field plots may represent insurmountable barriers for migrating species. Therefore it is recommended to subdivide large-sized field plots with hedgerows, to install marginal strips as recuperating areas for migrating species, or to convert high-input into low-input agriculture (organic farming). Organizations controlling organic farming suggest 1 year of biofarming as conversion time (regulation of the European Union), or 2–5 years (Bioland or Demeter regulation, respectively) before certified products can be sold.

Sunlight and fertilization move agricultural production systems away from the thermodynamic equilibrium. The introduced energy and all other avenues available in the system can now be utilized to counter the applied gradients and to oppose further movements away (restated second law of thermodynamics).^{72,102,170} Helpful in reaching and conserving a new stability are energy influxes (primary factor) and evolutionarily developed admirable microfloral and faunal recovering and surviving strategies (high elasticity). Also the capability of soil organisms to work together helps to react on changing conditions (sections 18.2–18.5; chapters 6, 12, and 15 in this volume). Earthworms may play an exhibited role in activating the soil microflora (chapter 14 in this volume; section 18.6.3). They shred the ingested physical (silt, clay) and chemical (humus, fresh organic material) soil particles and mix them during the gut passage intimately with the simultaneously taken-up bacteria, fungi, protozoa, nematodes, and tardigrada (chapters 5–13 in this volume). Shredding improves the nutrient availability for the earthworm and the ingested microflora. The microflora and fauna passing the earthworm gut are disseminated over soil depth and space with the excrements, which provide excellent starting conditions and enable the single parts to interact intensively with each other.⁹ Farmers try to copy the earthworm's role with diverse cultivation strategies without being able to avoid harm to the soil microbial and faunal community (chapters 1, 5–14 in this volume; section 18.7.1). Farmers realize this and experiment with soil life-protecting concepts (e.g., direct seeding techniques) (chapter 1 in this volume). Despite the impact of farming on agroecosystems, reduced and less diverse structured communities, reach similarly high yields (biomass productions; table 18.5) as so-called natural ecosystems with a highly diverse microflora and fauna organized in complex structures and hierarchial levels.^{70,71,135,136} In fields where farmers stop fertilization but continue in cropping soil depletion and low yields are progressing, meaning that nutrient losses have to be compensated. This observation of Justus von Liebig about 200 years ago has meanwhile been confirmed by various long-term field experiments (chapter 20 in this volume).²

Thorough soil mixing by ploughing, harrowing, organic, and mineral fertilization changes not only the soil structure, it also introduces oxygen and enhances organic matter degradation (chapters 1 and 3 in this volume; preceding sections in this chapter). Energy transferred into work and heat becomes increasingly available. Heat changes the entropy of a system, work does not. Entropy development and energy flows through an ecosystem can be measured and partitioned only at free Gibbs values around the thermodynamic equilibrium where the reaction goes in both directions and is reversible.^{114, 86} At high heatings the reaction goes only in one direction and is irreversible. Developing entropy can be calculated by $dS = dQ(\text{rev})/T$ (dQ = a tiny little bit of heat; rev = reversible; T = temperature in °Kelvin). Entropy measurements in real ecosystems require an extremely careful calometry and are not easy to carry out. Differences between captured and reradiated solar energy (black body temperature) provide information about disorder or randomness in an ecosystem. A cold, black body temperature corresponds with a high degree of order and stability.^{70,114,127,170} Ecosystem studies looking worldwide for entropy behaviors, biological diversities, and regeneration velocities^{55,56,70–72} and the bacterial partners of leguminous plants, the rumen or earthworm gut microflora, which funnel energy efficiently into their own production and reproduction, give hints on how agroecosystem stability could be recreated after perturbations (preceding sections in this chapter). Long-term experiences reveal that agricultural systems incorporating, for

example, N_2 -fixing plants, into the crop rotation are more robust against stress and more fit in supporting sustainability because they develop in a way that at least the amount of energy, carbon, and nitrogen removed from the system through harvesting, leaching, respiration, or denitrification is returned through photosynthesis and N_2 -fixation (sections 18.6.1–18.6.3).¹⁵⁴ Fertilized, animal-manured, or earthworm-enriched systems¹¹⁶ should be similarly able to compensate for stress, because agricultural plots cut off from energy and nutrient inputs retreat to configurations with lower energy degradation potentials and to earlier successional stage ecosystems (chapter 1 in this edition).^{114,102,70,135,73}

Living structures are open systems. They go through constant cycles of birth, development, regeneration, and death. With the influx of energy they grow and dissipate heat. The amount of released heat seems to correlate with the energy yield of the process. At changing conditions the preexisting organization is shattered. At such moments, “bifurcations points” systems desintegrate,¹⁴⁴ end in chaos, or leap to a new higher ordered level (section 18.2). New informations received at such moments from outside (e.g., genetic information) help to counteract changing conditions. Strategies connecting the soil microflora to the genetic storehouse of others for a rapid information exchange are conjugation, transduction, or transformation. In this context viruses play a crucial role, as DNA microarrays reveal (section 18.3).^{131,142,159}

Events shattering existing structures are, for example, longer drought periods. Drought resistance experiments in grassland plots cropped to an increasing number of plant species (from 1 to 32 species) revealed that the plots poor in species exhibited the highest resistance against drought, while plots with higher grades of biodiversity exhibited the highest biomass production.¹⁴⁵ Droughts seemingly select and enrich organisms with a high elasticity in managing less accessible water resources (chapter 13 in this volume) while others are disappearing.^{19,101,133,135,136} Soil bacteria react on water and other stresses with biofilm formation (section 18.5; chapter 7 in this volume). *Pseudomonas fluorescens* starts to produce alginate when surfaces dry out.²⁰ Alginate enables aggregation and by forming a biofilm the survival of non-spore forming bacteria increases significantly (chapter 7 in this volume). Increasingly understood is that before biofilm formation occurs a threshold concentration of the communication signals is required (section 18.5). How many communication signals are involved and must be concentrated before a perturbed agricultural production system can restabilize is far away from being understood. Species rapidly forming biomass by transferring small molecules as methanol, H_2 , NO_3^- , ethanol, CH_4 , N_2 , NH_3 , O_2 , CO_2 , or H_2O (gas) with virtual molar entropies measured at 25°C of 127, 130.6, 146, 161, 186.2, 191.5, 192.5, 205.1, 213.7 and 220 $J\ mol^{-1}\ K^{-1}$, respectively, into polymeric, less dissipative structures will minimize the entropy in the system.² Gaseous H_2O converted into its liquid form reduces entropy in a system by about 314% or from 220 to 70 $J\ mol^{-1}\ K^{-1}$.¹³² With the conversion of CO_2 with a virtual molar entropy of 213.7 $J\ mol^{-1}\ K^{-1}$ into polymer structures, in extreme, into graphite or diamond with entropies of 5.7 or 2.8 $J\ mol^{-1}\ K^{-1}$, respectively, can be entropy reductions reached of about 3750% (graphite) or about 7632% (diamond).^{2,132} Anabolic cycles that are strongly intertwined with catabolic pathways that provide additional energy (ATP) but simultaneously disperse heat and small molecules are behind the above entropy reductions (chapter 16 in this volume). Both types of processes, if balanced and intertwined, limit the energy flow into a potentially strong consumer–resource interaction or runaway consumption.¹²⁷ They serve to generate negative covariances between resources and enable a stabilizing effect at the population and community level. More precise predictions of balanced soil anabolic and catabolic ratios and richness-function effects should be aimed for in the future by advanced calorimetry combined with DNA microarray techniques, the counting of the present microbial and faunal individuals, estimates of nutrient availabilities, biomass-C, -N, -P, released metabolites, and plantal growth parameters (chapters 16–20 in this volume).^{136,137} Richness-function effects are observed in the more sessile communities of plants and microorganisms but seemingly not in the soil animal community.⁵⁵

Plants in self-organized systems grow in assemblies of many species to increase the leaf area for optimizing energy capturing through the chloroplast (section 18.3).⁷² Gross energy budgets of

terrestrial plants show that the vast majority of the captured energy is used for biosynthesis. The major dissipative pathway during plantal growth is evapotranspiration. Per gram of fixed photosynthetic material 200–500 grams of water or 2,500 joules per gram of water are transpired.⁷² At the equator, where 5/6 of the earth's solar radiation occurs, species diversity and trophic levels are vastly greater than elsewhere documents causal links between energy input, dissipative processes, and biodiversity.^{39,72} Next to energy transformation as a primary factor, geographical aspects (latitude, altitude), predation, competition, soil architectural heterogeneity, successional status, or the distribution of propagating units play powerful roles in biodiversity shaping.^{39,72} Soil pore diversity determines the number of niches (chapter 3 in this volume)¹⁶⁸ and nutrient gradient diversity decides about the number of types in a metabolic active soil community (chapter 16 in this volume).^{101,142} At changing conditions through farming, species adapt fastest that combine rapid generation times (intrinsic growth rate, r) with strategies for rapid genetic material exchanges and capabilities to grow on the expense of several organic compounds by lowering the threshold concentration significantly (K-strategists; chapter 16 in this volume). Another important advantage in counteracting changing conditions is working together as coculture experiments with the denitrifying fungi *Fusarium oxysporum*³⁰ and the denitrifying bacterium *Pseudomonas stutzeri* clearly show.¹¹⁰ Coculture convert *F. oxysporum* and *P. stutzeri*, accumulating nitrite and N_2O much faster into biomass (protein) and the non-greenhouse gas N_2 than by the sum of both pure cultures.¹¹⁰ The cooperative removal of the toxic nitrite benefit all inhabitants of an environment. Savanna forest sites receiving rainfalls after rather long drought periods are another example of cooperative success.¹⁷⁵ Despite long-lasting droughts, a rewetted Savanna soil can reactivate N-mineralization. Ammonium- and nitrite-oxidizing population densities increase by 42 and 145 times, respectively. Seemingly all kinds of stressed biotops either severely as agroecosystems or the Savanna soil or less impaired as shifting cultivation return to soil-specific activities and satisfying (wood) yields¹⁷⁵ when water, nutrient energy, and electron acceptors are sufficient and balanced. The self-organized returning of numerous coevolved plants, animals, and microorganisms to an original-like fertility in natural successions² results in the highest biomass productions (table 18.5), which can be approached by monocultured agroecosystems with specifically changed biodiversities (chapters 5–14 in this volume) or even by nutrient-balanced hydroculturing.

POSTSCRIPT

To understand the functioning of biodiversity in agricultural production systems is a significant challenge for physicists, biologists, ecologists, agriculturists, and mathematicians. A substantial proportion of variation in species richness can be explained statistically in terms of a few environmental variables. This is, however, far from a predictive theory of species richness resulting in an understanding of (agro)ecosystem stability and the richness of synergisms in nature.^{71,133,145} Complex mechanisms of genetic interaction between the genomes are suggested. Little to nothing is known about such integrated genome regulation, but this area may prove to generate exciting results in the future. For example, the existence of messaging molecules and integrated signal transduction systems from microbe to microbe in biofilms, or from amoebiae to nematodes in soils, or from legumes to rhizobia in the rhizosphere, or from the intestinal microflora to its host, or from ants to their fungal cultivars, and vice versa, are predicted to have evolved to integrate the mentioned interacting genomes. Horizontal gene transfer under bacteria allows to evolve in quantum leaps and is therefore the major driving force of bacterial adaption. The importance of horizontally transferred genomic islands for the development of virulent or symbiotic traits of bacteria is revealed by an increasing number of studies.¹⁴⁴ Analysis of such signal transduction systems, as well as underlying gene regulation, should reveal much about the manipulative power that synergistic systems can exert over each partner and thus should elucidate the mechanistic bases for modeling and predicting conflicts at environmental changes. Such approaches will finally help to

unravel evolutionary dynamics and will bring light to the open question, Who is winning the coevolutionary conflicts in between interacting communities?

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19 Food Web Interactions and Modeling

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19.1 INTRODUCTION

Food web models and biogeochemical cycling models have played an important role in the study of agricultural ecosystems by framing questions, interpreting empirical outcomes, and conveying in numerical and visual form the complexity of interactions among species, soil organic matter and nutrients (Parton et al. 1983; Hendrix et al. 1986; Andr  n et al. 1990; Moore and de Ruiter 1991; de Ruiter et al. 1993, 1994). Three important observations that have emerged from these studies serve as the basis for this chapter. First, conventional agricultural management practices have resulted in significant losses of soil organic matter (Metherell 1992; Beare et al. 1994; Vitousek et al. 1997). Second, the patterning of nutrient flow and trophic interactions within the soil food web under conventional management practices differ from their counterparts in native or conservation management practices, in ways that favor the bacteria and their consumers over the fungi and their consumers. Third, coincident with the losses in organic matter and changes in food web interactions and nutrient flow is a decrease in stability as measured by mathematical representations derived from the interactions (Moore et al. 1993; de Ruiter et al. 1995; McCann et al. 1998; Neutel et al. 2002). This chapter focuses on these observations and with models offers propositions on how food web interactions, nutrient dynamics, and stability are related.

19.2 SOIL FOOD WEB INTERACTIONS

The models and approach we work with are derivatives of the model for soils of the North American shortgrass steppe developed by Hunt et al. (1987). The model was based on the connectedness,

energy flow, and interaction food web descriptions proposed by Paine (1980). The connectedness web depicts the trophic interactions among organisms. The energy flow web represents the flow of energy, biomass, or elements among organisms. The interaction web depicts the influences of the dynamics of one group on another. This approach has been adopted by several research groups that have attempted to link the structure of soil food webs in relation to the decomposition of organic matter and the mineralization of nutrients (Andr  n et al. 1990; Brussaard et al. 1988; Brussaard et al. 1997; de Ruiter et al. 1993; Hendrix et al. 1986; Hunt et al. 1987; Moore et al. 1988).

19.2.1 THE CONNECTEDNESS FOOD WEB

The connectedness web defines the model’s basic structure (fig. 19.1). The diagram simplifies the high diversity and complexity within soils by defining the web in terms of functional groups of organisms that shared similar prey and predators, feeding modes, life history attributes, and habitat preferences (Moore et al. 1988). At the base of the web are plant roots, labile (C:N ratio < 30:1) and resistant (C:N ratio > 30:1) forms of detritus, and an inorganic nitrogen source. These basal resources are utilized by microbes and invertebrate consumers, terminating with a suite of predators.

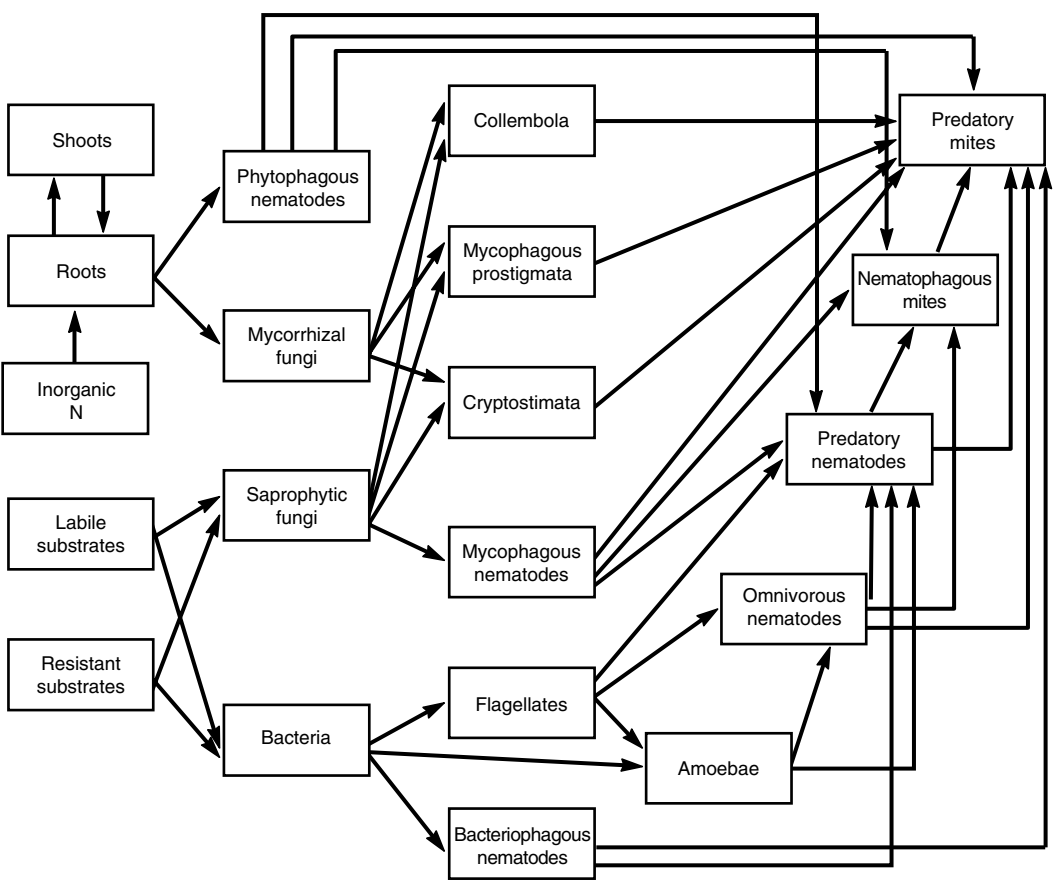


FIGURE 19.1 Diagram of the belowground food web for the North American Shortgrass Stepp (Hunt et al. 1987). Species are aggregated into functional groups based on food choice, feeding mode, habitat selection, and life-history parameters (Moore et al. 1988). Detritus represents all dead organic material (Moore et al. 2004). Material flows to the detritus pool that result from death, excretion of waste products are not represented in the diagram. The biomass estimates (g C m⁻²) are presented under the functional group’s name. The flux rates (g N m⁻² yr⁻¹) from resource/prey to consumer/predator are presented above the arrows. The interaction strengths are presented below the arrows (g C m⁻²)⁻¹ yr⁻¹.

The assignment of species to functional groups is not an exact science. Two common criticisms of the assignments are that they are not uniformly applied across taxa, and that they oversimplify the diets of many organisms. Current renditions of soil food webs tend to lump all prokaryotes and archaea into a single functional grouping of bacteria, while arthropods are parsed into several functional groupings. This discrepancy in detail is not limited to soil food webs, as most described webs exhibit greater resolution at higher trophic levels than lower levels, with predators often being represented by a single genus or species and basal resources represented by much courser categories (e.g., algae, vascular plants, and detritus). Functional groups often do not reflect the full breadth of its constituent species' diet. Collembola feed on detritus, algae, pollen, and fungi, yet are categorized as mycophagous, while many species of amoebae consume fungi, yet all are categorized as bacterial feeders. Obvious shortcomings can be resolved by modifying the description, but short of describing the interactions of every species, some simplification is inevitable, otherwise the utility of the description diminishes. What these criticisms and discrepancies illustrate are needs for better descriptions, and cautions to not overinterpret the models or apply them too broadly.

19.2.2 THE ENERGY FLOW FOOD WEB

The energy flow web expresses food web structure in quantitative measures of population sizes (biomass) and feeding rates or fluxes (fig. 19.1). Estimates of population size are obtained from field samples, preferably at several times to capture temporal dynamics or to estimate steady-states or long-term averages (Moore et al. 1996). Table 19.1 provides estimates of how flow are derived indirectly using the connectedness food web model, in conjunction with estimates of population

TABLE 19.1
The Estimated C:N Ratios, Turnover Rates, Assimilation Efficiencies, Production Efficiencies, and Population Sizes for the Function of the Soil Food Web for the North American Shortgrass Steppe

Functional group	C:N	Turnover rate (g C m ⁻² yr ⁻¹)	Assimilation efficiency %	Production efficiency %	Biomass (mg C m ⁻² yr ⁻¹)
Predatory Mites	8	1.84	60	35	0.160
Nematophaous Mites	8	1.84	90	35	0.160
Predatory Nematodes	10	1.60	50	37	1.080
Omnivorous Nematodes	10	4.36	60	37	0.650
Fungivorous Nematodes	10	1.92	38	37	0.410
Bacteriophagous Nematodes	10	2.68	60	37	5.800
Collembola	8	1.84	50	35	0.464
Mycophagous Prostigmata	8	1.84	50	35	1.360
Cryptostigmata	8	1.20	50	35	1.680
Amoebae	7	6.00	95	40	3.780
Flagellates	7	6.00	95	40	0.160
Phytophagous Nematodes	10	1.08	25	37	2.900
AM-Mycorrhizal Fungi	10	1.20	100	30	7.000
Saprobic Fungi	10	2.00	100	30	63.000
Bacteria	4	1.20	100	30	304.000
Detritus	10	0.00	100	100	3000.000
Roots	10	1.00	100	100	300.000

Note: The values are used to calculate carbon and nitrogen fluxes, F_p (eq. 19.1), egestion rates, rates of mineralization, and the elements of the Jacobian matrix, α_{ij} (eq. 19.2).

From figure 1, Hunt et al. (1987).

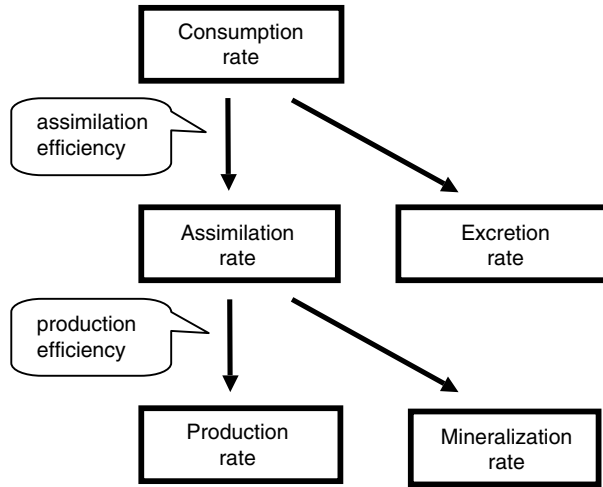


FIGURE 19.2 Schematic diagram of the fractionation of the fate of consumed biomass from resource/prey to consumer/predator.

sizes, turnover rates, consumption rates, prey preferences, and energy conversion parameters (de Ruiter et al. 1993; Hunt et al. 1987; O'Neill 1969). Though coined an energy flow description, more often than not, the description depicts elemental flows, usually C or N, estimated from biomass, and known elemental ratios of the taxa.

Feeding rates were estimated using the procedures presented by Hunt et al. (1987). Consumed matter is divided into a fraction that is immobilized into consumer biomass (assimilation) and a fraction that is excreted to the environment as feces, orts, and unconsumed prey, and of the assimilated fraction, material that is incorporated into new biomass (production) and material that is mineralized as inorganic material (fig. 19.2). The estimates begin with top predators with the assumptions that the amount of material required to maintain the predator's steady-state biomass must equal the sum of its steady-state biomass and loss due to death divided by its ecological efficiency:

$$F = (D_{nat}B + P) / e_{ass}e_{prod} \quad (19.1)$$

where F is the feeding rate (biomass time⁻¹), D_{nat} is the specific death rate (time⁻¹) of the consumer, B (biomass) is the population size of the consumer, P is the death rate to predators (biomass time⁻¹), and e_{ass} and e_{prod} are the assimilation (%) and production (%) efficiencies, respectively. For a top predator the death due to predator is zero. For predators that consume multiple prey-types the fluxes are weighted by the predators feeding preferences for the respective prey. The estimation procedure moves downward through the prey to the basal resources with fluxes to each prey, taking into account the biomass lost to predation. A dynamic version can be constructed by taking into account changes in the biomasses over an interval of time t (i.e., adding $\Delta B/t$ to the numerator of eq. 19.1).

The energy flow description provides a snapshot of dominant fluxes of C, N, or energy. This in and of itself can be a useful exercise, providing critical information to those in applied fields and theoreticians alike (see below). The description led to the identification and formalization of an even coarser depiction of the food web that was based on dominant flows of energy through plant roots and its consumers, bacteria and its consumers, and fungi and its consumers (Hunt et al. 1987; Moore and Hunt 1988). Both empirical and theoretical treatment of food webs and trophic interactions had anticipated these pathways or energy channels. Coleman et al. (1983) noted that within the rhizosphere, the dynamics of the transfer of C and N among plants and soil biota was

mediated by a fast cycle dominated by bacteria and their consumers and a slow cycle dominated by fungi and their consumers. May (1972) had proposed on the grounds of dynamic constraints that the arrangement of species into interacting blocks was a more likely arrangement as opposed to a random network of interactions. The melding of the energy flow description with the dynamic properties is discussed below and will play an important part in linking the changes in the structure of the soil food web with nutrient dynamics and stability (de Ruiter et al. 1995; Moore et al. submitted).

19.2.3 THE INTERACTION FOOD WEB

The interaction web emphasizes the strengths of the per capita effects, or interaction strengths among the functional groups (i.e., the dynamics of one group on the dynamics of another group). The interaction strengths can be derived via manipulative experimentation (Paine 1992) or from the biomass estimates of the functional groups, the estimates of energy flow, and the elements of the Jacobian matrix obtained from the series of differential equations used to describe the dynamics of each functional group (Moore et al. 1993; de Ruiter et al. 1995). There are merits and downsides to both approaches (Wootton 1997), but we have opted for the latter.

The elements of the Jacobian matrix are the interaction strengths, which are either zeros to denote no direct interaction (feeding) and non-zeros to represent direct interactions between functional groups. The elements are the partial derivatives of the equations describing the growth and dynamics of the functional groups at or near equilibrium (May 1973):

$$\alpha_{ij} = [\delta(dX_i^* / dt) / \delta X_j] \quad (19.2)$$

where α_{ij} refers to the interaction strengths that designate the per capita effects (in the case of the present energy flow webs per biomass effects) of the functional groups upon one another. Interaction strengths can be derived directly from equations used to model the population dynamics of the functional groups if the population densities of the functional groups and the feeding rates (eq. 19.1) are known. The key assumptions behind the estimation procedure are as follows: (1) the equilibrium biomass of functional group i (X_i^* in eq. 19.2) in the rate equations are approximated from long-term seasonal averages of biomass of functional group i (B_i), and (2) the consumption terms in the rate equations for prey i to predator j can be estimated from the flux rates estimated as described in equation 19.1 (i.e., $F_{ij} = c_{ij} X_i^* X_j^*$), where c_{ij} is the coefficient of consumption of functional group j on functional group i . Hence, if derived from rate equations based on Lotka-Volterra rate equations, the interaction strengths are $\alpha_{ij} = F_{ij}/B_j$ for the per capita effect of predator j on prey i , and $\alpha_{ji} = a_{ji}F_{ij}/B_j$ for prey i on predator j (Moore et al. 1993; de Ruiter et al. 1994; de Ruiter et al. 1995). The diagonal elements of the matrix cannot be derived from field data or estimates of energy fluxes, but can be scaled to the specific death rates (de Ruiter et al. 1995) or can be set at levels that ensure stability (Neutel et al. 2002).

The interaction web has both descriptive and theoretical utility. As within the energy flow web, the estimates of interaction strength do provide a snapshot of sorts, with a caveat. The magnitude of the flows within the energy flow description equate to the importance or contribution of the flows to the overall flow of energy within the system. The magnitude of the interaction strengths do not necessarily equate to their importance to stability (Paine 1988), as weak interactions, both in terms of energy flow and per capita effects, can have profound effects on stability (McCann et al. 1998). The patterning of the interactions and their relationships among one another appear to be more important than the magnitude of the interactions per se (de Ruiter et al. 1995; Neutel et al. 2002).

De Ruiter et al. (1995) studied the patterning of the interactions within seven soil food webs using a variant of the approach advocated by May (1972). While May (1972) manipulated the connectedness structure and hence the Jacobian matrices of food webs that varied in diversity and in the number and positioning of interactions to study stability, de Ruiter et al. (1995) preserved

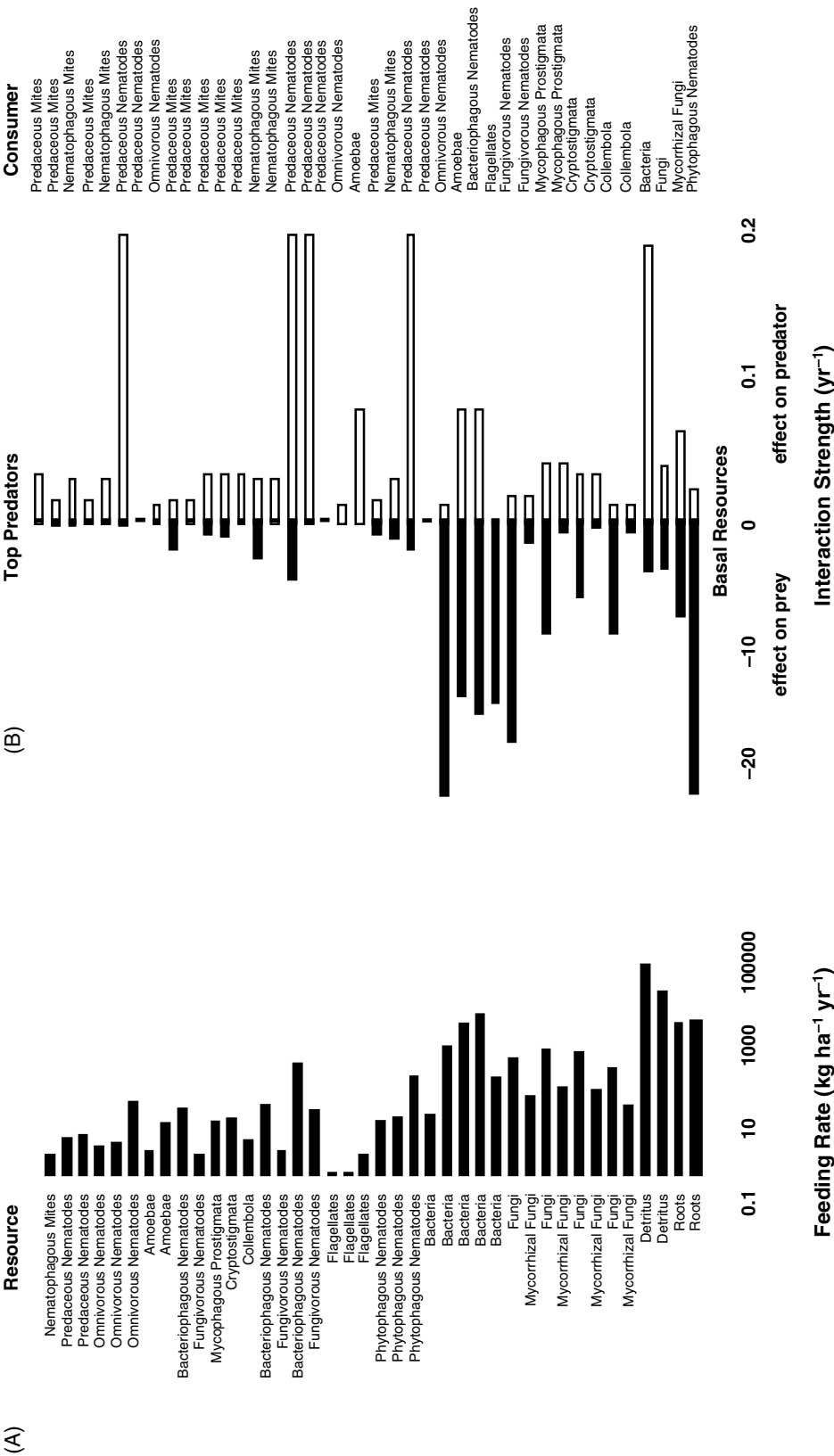


FIGURE 19.3 The patterning of the (A) feeding rates estimated using equation 19.1, and (B) interaction strengths estimated using equation 19.2 with trophic position (basal resources at bottom to top predator at top) for the food web of soils from the conventional tillage plots at the Lovinkhoeve Experimental Farm, Marknesse, Netherlands. Resource/prey are presented on the left and consumers/predators are presented on the right. Adapted from de Ruiter et al. (1995).

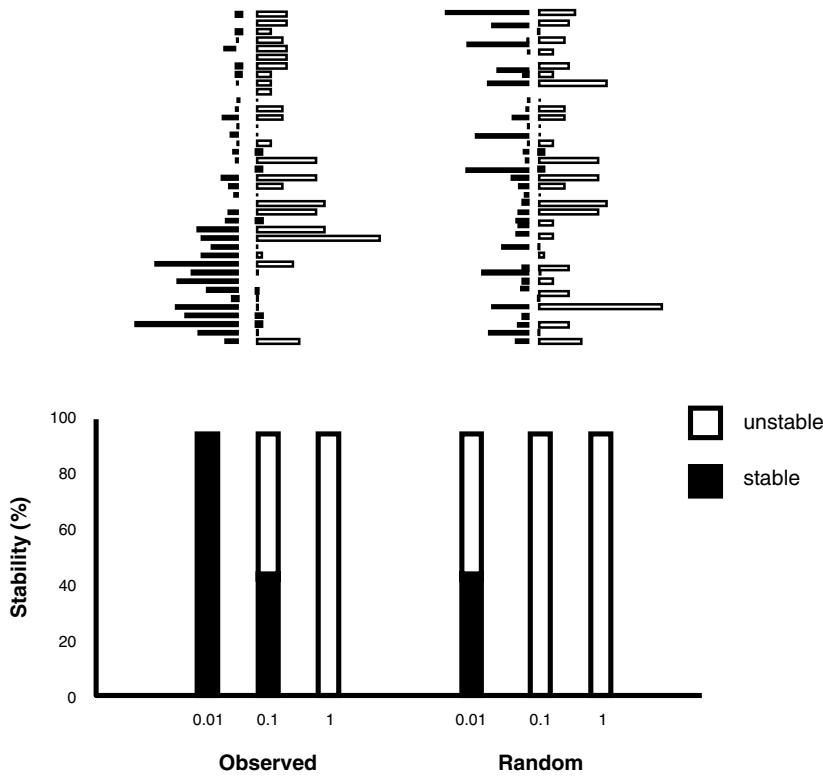


FIGURE 19.4 Monte Carlo trials comparing the observed food webs that possessed the patterning presented in figure 19.3 (caricature above observed graph) to random food webs of similar structure but for whose interaction strengths were transposed at random (caricature above random graph). For the observed food webs, the interaction strengths were sampled from uniform distribution $[0, 2\alpha_{ij}]$, where α_{ij} were estimated from equation 19.2 using field data and the life-history parameters in table 19.1. The intragroup interference term α_{ii} were set to the death rates scaled at three levels of magnitude (0.01, 0.1, and 1.0). Adapted from de Ruiter et al. (1995) and Moore and de Ruiter (1997).

the diversity and the interactions within the web (i.e., preserved the connectedness structure and positioning of the non-zero elements), but transposed the paired elements of the Jacobian matrix with other pairs. The observed food webs possessed a distinct asymmetry in the pattern of interaction strength when arranged by trophic position. The effects of prey on predators is large at the base of the food web and decrease with increased trophic position, while the converse is true for the effects of predators on prey (fig. 19.3). The pattern was preserved within the energy channels (Moore and de Ruiter 1997). The webs that possessed the observed patterning of interaction strengths were more stable than those that possessed the manipulated arrangements (fig. 19.4).

19.3 MODELING THE IMPACTS OF AGRICULTURAL PRACTICES

Agricultural management practices affect the densities of soil biota, the availability of plant limiting nutrients, and the quantity and quality of soil organic matter (Wardle 1995; Beare 1997; Vitousek et al. 1997; Doles et al. 2001). Our aim is to connect these changes in an integrative manner. We begin with a discussion on how one aspect of agricultural management, tillage, affects soils and soil organic matter, followed by a discussion of its effects on soil biota and food webs.

19.3.1 EFFECTS OF TILLAGE ON SOIL ORGANIC MATTER AND NUTRIENT DYNAMICS

The physical process of tilling soils over several years disrupts the distribution of soil organic matter within soil horizons, and alters the structure of soil that develops naturally. Conventional tillage methods overturn soils, mixing dead plant material and surface litter and topsoil with lower layers, and in the process alters the temperature and moisture regimes of the materials and layers. The physical action and exposure also alters the aggregate structure soil, often disrupting the larger size fractions to smaller ones, thereby exposing occluded materials to biota and weathering (fig. 19.5). Over time tillage, coupled with the addition of inorganic fertilizers and crop removal, creates an environment whereby the organic materials that were held within the aggregates are mineralized by soil microbes, resulting in a net loss (Vitousek et al. 1997; Wander and Bidart 2000).

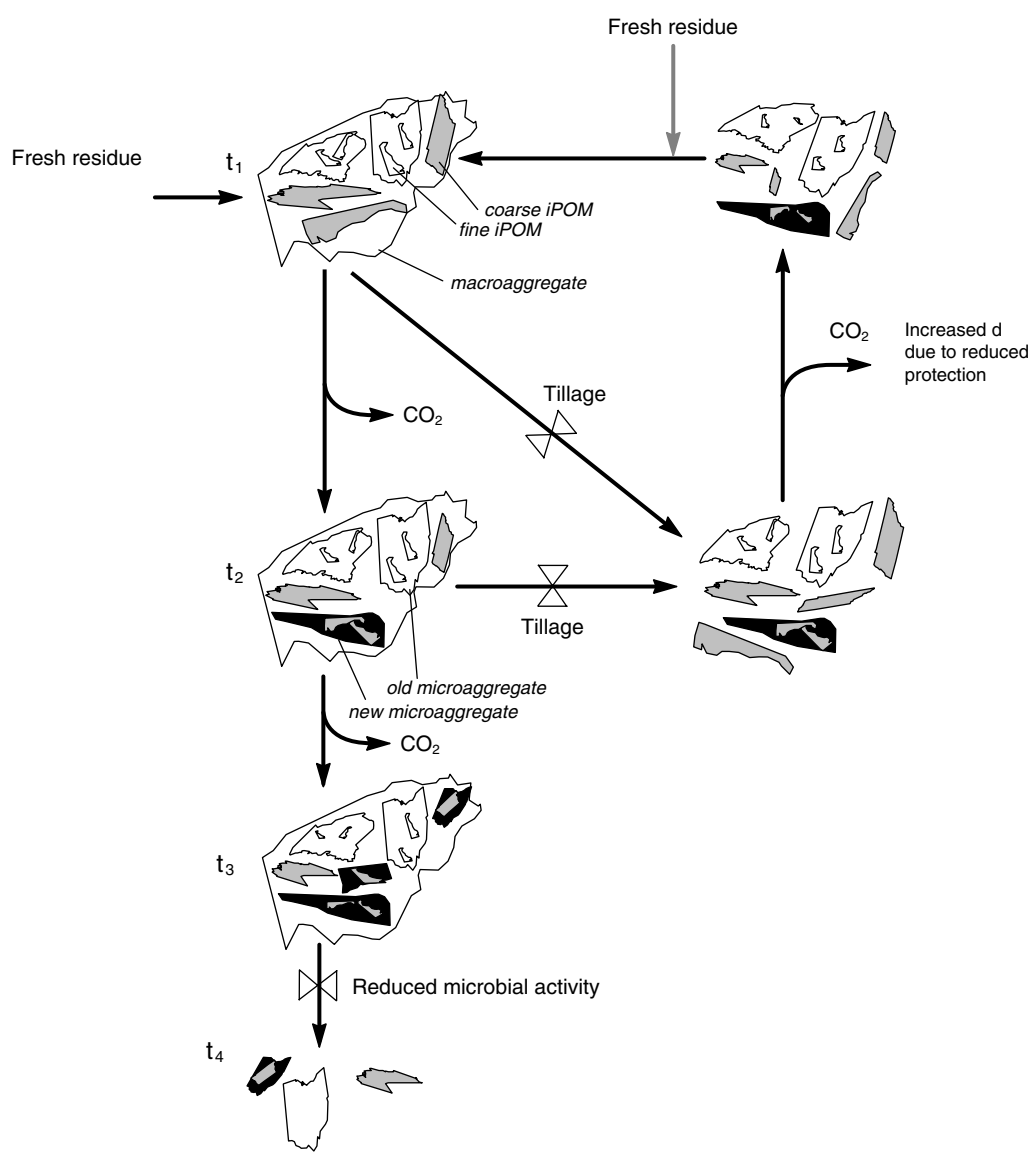


FIGURE 19.5 Aggregate turnover model or “life cycle” of a macroaggregate (Six et al. 2000). Carbon in the form of particulate organic matter, POM-C, is protected within the macroaggregate structure, but when the system is disturbed (i.e., through conventional tillage), POM-C is no longer protected within the macroaggregate and is mineralized through microbial action.

Management practices that rely on less intrusive tillage practices (no-till and stubble-mulching) and organic fertilizers have ameliorated this trend (Elliott et al. 1984; Brussard et al. 1988; Elliott and Coleman 1988; Beare et al. 1994; Frey et al. 1999). No-tillage and other reduced tillage practices leave plant residues on the soil surface and are far less disruptive to the aggregate structure (Six et al. 1999; Wander and Bidart 2000). Several authors have reported a higher percentage of large aggregate fractions within soils under no-till management compared to those under conventional management (Beare et al. 1994; Six et al. 1999; Simpson et al. 2004; fig. 19.6).

The current biological explanation for the changes in soil organic matter concentration and the distribution in size classes of soil aggregates being vetted revolves around the relative activities and contributions of soil bacteria and fungi to the formation and mineralization of the soil carbon pool (Tisdall and Oades 1982; Cambardella and Elliott 1994). Work in the past decade has focused on the contributions of microbial-derived carbon in the form of bacteria and fungal-specific amino sugars to soil aggregate formation. The ratio of the fungal-derived amino sugar glucosamine (Glc) to the bacterial-derived muramic acid (MurA) has proved to be a useful indicator of the relative importance of fungi to bacteria (Zhang et al. 1998). Fungi and their by-products are believed to have a dominant role in the formation of large and intermediate-sized aggregates (Cambardella and Elliott 1994). Soil aggregates from soils under no-till management tend to have higher Glc:MurA ratios than their counterparts collected from conventionally tilled plots (Guggenberger et al. 1999; Frey et al. 1999; Simpson et al. 2004).

19.3.2 EFFECTS OF TILLAGE ON FOOD WEB STRUCTURE AND STABILITY

Models of soil foods have captured the responses of soil biota and the mineralization of carbon and nitrogen with surprising accuracy and have revealed important patterns (de Ruiter et al. 1994; Schröter et al. 2003). A common response to intensive tillage has been an increase in soil respiration, an increase in nitrogen mineralization rates at the shoulders of the growing season, and the demise of biomass and activity within the fungal pathway and an increase in the biomass and activity in the bacterial pathway (Elliott et al. 1984; Hendrix et al. 1986; Andrén et al. 1990; Moore and de Ruiter 1991;

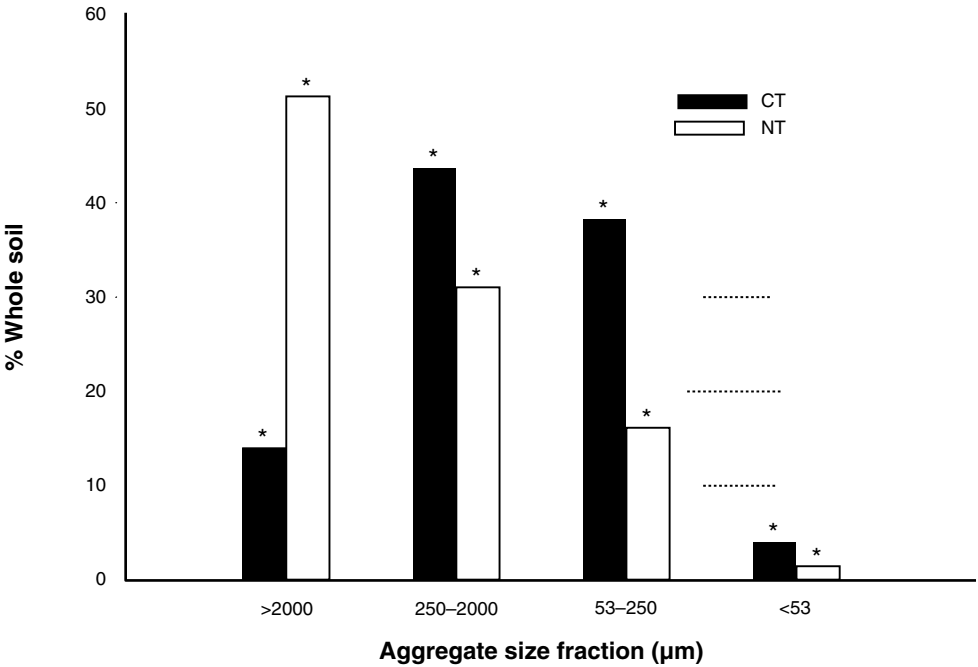


FIGURE 19.6 Water-stable aggregate size distribution at 0–5cm depth from CT/NT comparison plots, Horse-shoe Bend, GA (Simpson et al. 2004). “*” Indicates significant difference between tillage treatments ($p < 0.05$).

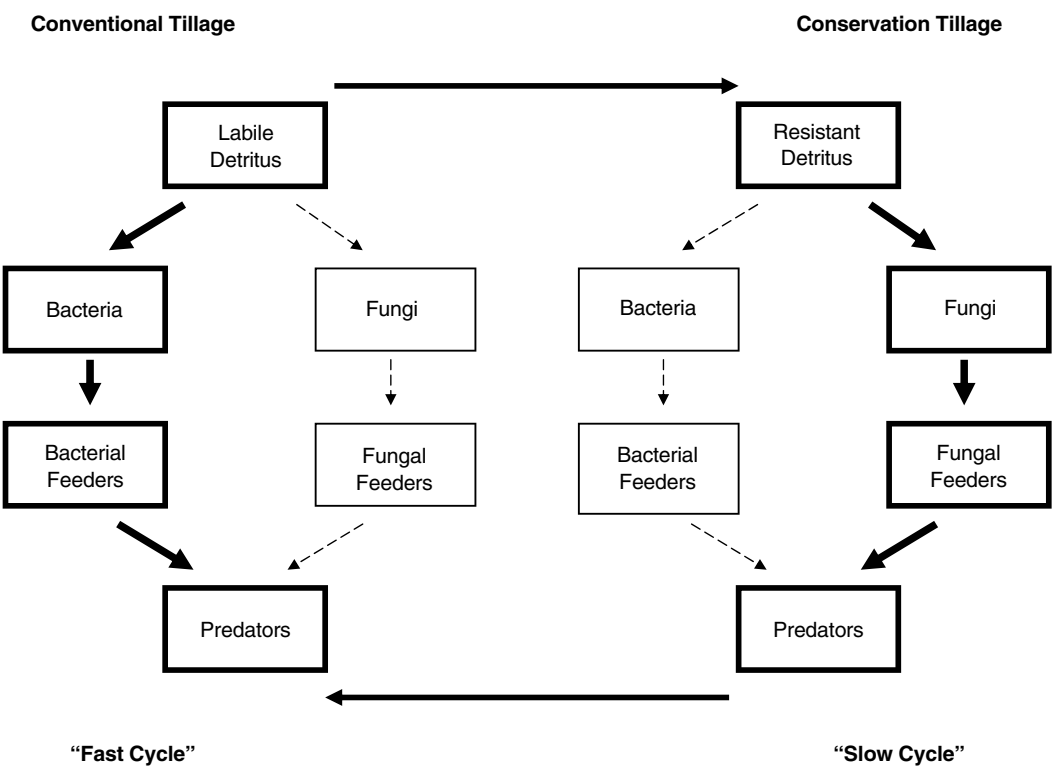


FIGURE 19.7 Simplified representations of the bacterial and fungal energy channels under conventional tillage and no-till or reduced tillage systems. Several studies have demonstrated that the placement of crop residues, the bioavailability of labile versus resistant soil carbon, the changes in aggregate structure, the relative sizes of habitable pore space, and water availability operate to induce shifts from one pathway to the other (Hendrix et al. 1986; Moore 1986; Andr  n et al. 1990; Beare et al. 1994; Moore and de Ruiter 1991).

Wardle 1995). Comparisons of the responses of soil food webs and selected taxa from soils within systems under conventional tillage compared to no-till or reduced tillage in terms of the placement of crop residues, the bioavailability of labile versus resistant soil carbon, the changes in aggregate structure, the relative sizes of habitable pore space, water availability, and the toxicity of pesticides confirm this conclusion (fig. 19.7).

Apart from elucidating patterns, models have offered some insight into the mechanisms at play, and the long-term consequences as they relate to sustainability. Coleman (1994) likened the detritus portion of the soil food web to the microbial loop of aquatic food webs, and in doing so, separated and compared the life history characteristics of the bacterial pathway from the fungal pathway, confirming their fast and slow cycling properties, respectively (Coleman et al. 1983). Apart from affecting cycling rates, the life history traits affect the resilience of the pathways to disturbances (Moore et al. 1993). Simple food chains parameterized to emulate a fast bacterial pathway to ones parameterized to emulate a slow fungal pathway had shorter return times (fig. 19.8). If we conservatively assume that the both pathways were equally affected by a tillage event (i.e., the same proportionate decline in biomass), then the models would predict that the bacterial pathway would rebound at a faster rate than the fungal pathway, offering one piece of the explanations for the observed shifts conceptualized in figure 19.4. Models of coupled bacterial and fungal pathways and whole food webs provide more realistic assessment of how changes in the relative sizes of energy flows or shifts from one pathway to another might affect food stability (fig. 19.9). Models of coupled bacterial and fungal pathways illustrate that the most stable configurations are ones where neither pathway completely dominates (Moore et al. 2004; Moore et al. submitted).

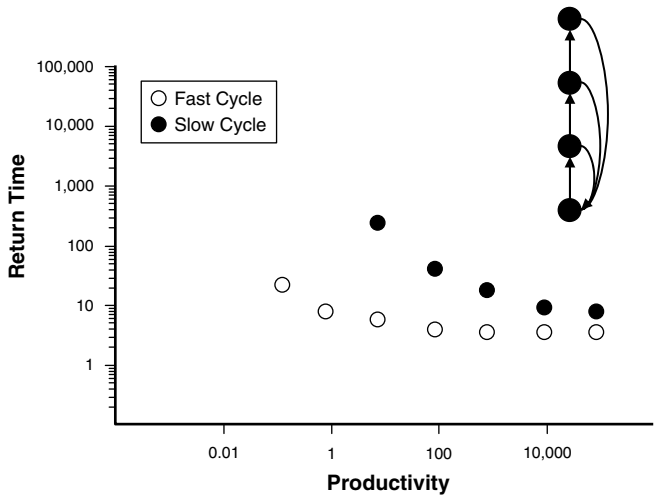


FIGURE 19.8 A comparison of the return-times (sensu Pimm 1982) of fast (open symbols) and slow (closed symbols) cycling of four species detritus-based food chains (redrawn from Moore et al. 2004).

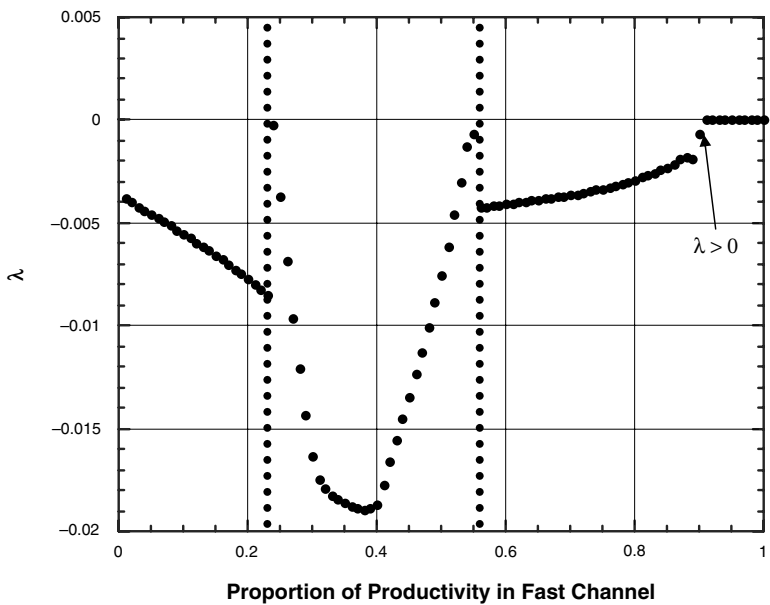


FIGURE 19.9 The dominant eigenvalues for a series of simulations of a model representing coupled fungal-based and bacterial-based pathways linked by a common predator. The microbes utilize a common resource. The equations and parameters used in the simulations are presented in Moore et al. (2004). The x-axis represents the proportion of resources passing through the fast cycling bacterial-based pathway (p), while the y-axis represents the dominant eigenvalue (λ) for the system at each level of p . The dashed vertical lines represent unstable transitions. The most stable configuration occurs when the two pathways are coupled, and unstable if most of the energy passes through the fast pathway ($\lambda > 0$). Adapted from Moore et al. (2004).

19.4 DISCUSSION AND CONCLUSIONS

The empirical studies and modeling exercises lead to a series of observations and propositions that help link soil food web structure, nutrients dynamics, and stability.

1. The first observation is that the utilization of resources by species is highly diverse yet compartmentalized. The compartments are organized as a series of quasi-independent interacting energy pathways. The pathways are made up of species that share similar habitat requirements and complimentary life history traits. As a result of this, matter is processed and energy is transferred within the pathways at distinct rates. In soils, these pathways originate from plant roots and detritus. The detritus pathway is divided into a bacterial pathway and a fungal pathway.
2. The second proposition is that the soil decomposer communities are tightly coupled through the quality of plant litter and soil organic matter, and shared life history characteristics. Fungi and bacteria have affinities toward substrates that differ in quality and contribute to the formation and degradation of soil organic matter.
3. Disturbances alter the bacterial and fungal pathways to differing degrees and hence the pattern of the flow of energy within ecosystems. Nutrient availability to plants and retention within the ecosystem are affected by changes in the activity of the pathways relative to plant growth. In arable soils, tillage has a disproportionately large adverse affect on the fungal pathway compared to the bacterial pathway. Nutrient availability and retention were either tied to the activities of the fungal pathway, or were a function of the combined activities of the bacterial and fungal pathways, and how synchronous these activities are with plant growth.

What the modeling, field manipulations, and laboratory experiments suggest is that it is neither the size of the interactions nor the magnitude of the nutrient flow that determines the stability of an ecosystem. Rather, it is the patterning of the interactions among species and the distribution of nutrients within the community that governs its stability. The common feature that disturbances have on ecosystems, be they natural or human induced, is to change the densities of organisms, the species composition of communities, and hence the patterning of interaction and nutrient flows within them. It is through these changes that the stability of the system is impacted. In short, models have shown us that it is the energetic organization of communities that form the basis of ecosystem structure and stability.

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20 Soil Quality Assessment and Long-Term Field Observation with Emphasis on Biological Soil Characteristics

Hans-Rudolf Oberholzer and Heinrich Höper

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20.1 INTRODUCTION

Main objectives of the Convention on Biological Diversity (UNEP 1992) are the conservation of biological diversity and the sustainable use of its components. Biological diversity includes diversity within species, between species, and of ecosystems.

The soil is related to biological diversity under different aspects:

- The soil is a habitat and resource (e.g., of water and nutrients) for plants and, thus, a factor for natural plant biodiversity. In this context frequently the term “soil fertility” is used, which will be defined later. The relationship between soil and plant diversity will not be addressed in this chapter.
- The soil is a habitat for soil organisms, namely for the soil fauna and the soil flora and thus a determining factor for soil microbial and soil faunal diversity. This is one essential function of soils, and diversity of soil organisms is the property for measuring this function. Soil quality can be defined with respect to this habitat function.
- Soil organisms are essential parts of a lot of soil functions. Thus, biological diversity may be a prerequisite of soil functioning. Soil functioning (i. e., the degree of fulfillment of a set of soil functions with respect to a given land and soil use) can be defined as soil quality. Thus soil organisms and their diversity are part of soil quality.

This chapter discusses the aspects of biological diversity related to soil fertility and soil quality. Soil fertility and soil quality are subjects of modern soil protection legislation including the biodiversity-related aspects. Both are abstract concepts that are successively concretized by defining soil functions, soil properties, analytical parameters, and indicators referring to specific methods (section 20.2.2). The limitations of current methods and indicators on biodiversity to characterize soil with respect to biotic functions and soil quality are pointed out. This also concerns the frequently presumed relationship between biodiversity and soil functioning (section 20.2.2.5).

Soil quality assessment will be discussed with emphasis on timing and the choice of the adequate reference system (section 20.2.3). The specific value of information derived from biological soil characterization in soil quality assessment is shown (section 20.3). Practical aspects of soil quality assessment and soil monitoring with respect to microbial soil parameters are illustrated. Examples for the soil assessment using soil microbial biomass measurements are presented with the intention to show which requirements potential soil quality assessments based on biodiversity parameters have to fulfill (section 20.4). Finally, requirements for the use of biotic parameters in soil monitoring and the outcome of several years of the Lower Saxony Soil Monitoring Program are presented (section 20.5). Microbial biomass, although a “black box” parameter with respect to biodiversity, may be an appropriate parameter to characterize the soil state as a habitat and functioning of essential organisms based soil processes.

20.2 SOIL BIOLOGICAL DIVERSITY AND SOIL QUALITY

20.2.1 SOIL BIOLOGICAL DIVERSITY IN SOIL PROTECTION LEGISLATION

Only a few countries have established explicit soil protection legislation. In most cases soil protection aspects are included more general environmental laws. In Switzerland an Ordinance Relating

to Impacts on the Soil (OIS 1998) was realized based on the Law Relating to the Protection of the Environment of 1983. In Germany the Federal Soil Protection Act went into force in 1998 (BBod-SchG 1998).

The Swiss ordinance is based on the protection of soil fertility. According to the definition of soil fertility in this ordinance, a soil is fertile if

- it demonstrates a diversified and biologically active biocoenosis, a structure typical for its site and an intact capability for decomposition;
- natural or cultural plants and plant communities can grow and develop normally and their properties are not adversely affected;
- the foodstuffs and fodder produced on it are of good quality and do not present a hazard to the health of human or animals;
- ingestion or inhalation of it does not present a hazard to humans or animals.

This definition is quite general. It implies that not only highly productive, agricultural soils may be fertile but also other nonagricultural soils with low productivity. Moreover, a soil is fertile if its functions, respectively properties, are in a typical range.

In contrast, the German Federal Soil Protection Act is based on soil functions to be protected:

The following soil functions, involving the presence or the activity of soil organisms, are (among other functions) cited in the Act:

1. Natural functions
 - soil as a basis for life and a habitat for people, animals, plants, and soil organisms,
 - soil as part of natural systems, especially water and nutrient cycles,
 - soil as a medium for decomposition, balance, and restoration as a result of its filtering, buffering, and substance-converting properties, especially for groundwater protection.
2. Functions useful to man as
 - land for agriculture and forestry

Regarding agricultural land use a good agricultural practice is required by the precautionary principle in order to prevent hazards to the soil provoked by the land user. The following aspects of good agricultural practice concerning soil organisms and their habitat are explicitly mentioned:

Good agricultural practice means:

- to preserve and to enhance soil structure,
- to avoid soil compaction and soil erosion,
- to preserve elements of landscape necessary for soil conservation,
- to preserve and to enhance biological activity by crop rotation, and
- to preserve a site-specific carbon content of the soil.

Even if the concepts followed by the two regulations are quite different (i.e., the concept of “soil fertility” versus the concept of “soil functions”), soil organisms (i.e., soil biological diversity and activity) are explicitly protected by both approaches. On one hand, this means a protection of the habitat function of the soils (i.e., the role of the soil as a living space for a diverse biocoenosis). On the other hand, soil functions based on the activity of soil organisms have to be preserved (e.g., decomposition of organic materials, nutrient cycling, biological effects on growth and development of plants, control of plant diseases and degradation of pollutants). As a consequence, soil biodiversity is a subject of protection by itself and by the soil functions that are carried out by soil organisms. Both aspects are not easily put into soil protection practice. Financial resources are restricted and our knowledge on soil organism communities and their role in soil functioning is far from being clear. Thus, a complete conservation of up-to-date species composition is not possible. As the full

biological richness of soils is still ignored and evolution happens, it is not clear which type of species diversity has to be protected and to which degree.

Concerning the role of soil organisms as a carrier of soil functions, the relationship between biodiversity and a given soil function needs to be demonstrated.

There is a need for long-term field observations on changes either of soil species diversity or of soil processes and properties as carriers of soil functions under different land and soil uses.

20.2.2 SOIL QUALITY AS A SET OF FUNCTIONS AND PROPERTIES

The Convention on Biological Diversity (UNEP 1992) does include a sustainable use of the components of soil biological diversity. An evaluation of soils with regard to land use is a subject of the concept of soil quality. In this context, land use is defined in the largest sense as all nondestructive benefit of soils for the landowner and for the general public (e.g., including the role of soils in groundwater protection and in climate change). For application, the concept of soil quality has to be made operational. Therefore, the general term of soil quality is disaggregated into soil functions to be preserved. These are characterized by assessable soil properties.

20.2.2.1 The Terms Soil Fertility and Soil Quality

Patzel et al. (2000) made an extensive review of soil fertility in the literature of soil science, agronomy, and ethnic studies. They found that soil fertility is not applicable as a technical term in natural sciences as it describes a definite but elusive soil feature that it is not fully operational.

“Soil quality in contrast to soil fertility refers to sets of assessable soil attributes and functionalities, which are assigned by value judgments.” It is a tool that integrates different soil state variables and functions in order to evaluate the capacity of a soil to do what it is expected to do or to assess the sustainability of current land-use practices (Patzel et al. 2000).

For scientific or practical use soil quality is described as defined lists of soil functions or properties. Soil quality has to be related to human soil and land use (e. g., agriculture and forestry, fallow land or nature protection area, drinking water caption, or settlement area). Consequently, diverging from the concept of Doran and Parkin (1996), different soil qualities have to be defined depending on the current or intended principal soil use. There may even be conflicting definitions of soil qualities, for example, definition of soil quality for agricultural use (high fertility = high nitrogen content) or for drinking water caption (low risk for nitrate leaching = low nitrogen content). Different sets of soil functions are relevant for each soil quality.

In specific cases the protection of soil biological diversity may become the main objective of land use, for example, nature protection areas aimed at the conservation of soil organisms (Höper and Ruf 2003). In this case, soil quality has to be defined with respect to this specific objective.

But, in general, soil organisms and biological driven processes are only one component beneath other, abiotic components of soil quality.

20.2.2.2 Soil Functions

Soil function is defined as “the potential utility of soils in landscapes” to nature and mankind “resulting from the natural combination of soil chemical, physical, and biological attributes” (after Johnson et al. 1992, modified). Concerning soil biological diversity, soil does not provide but enables a specific diversity through its habitat function (table 20.1). Enabling the diversity is a function of the soil, which results from several soil properties, whereas the diversity of the soil organisms is a soil property (attribute) that again makes a contribution to soil functions. Additionally, biological diversity and activity are carriers of different soil functions, for example, decomposition of organic substances or nutrient cycling (table 20.1). Several lists of soil functions exist, for example, the German Soil Protection Act (cf. section 20.2.1).

TABLE 20.1
Soil Functions, Properties, and Assessable Parameters. Examples for Biological Soil Parameters.

	Function: Diversity of soil organisms	Function based on biological activity (e.g., Metabolization/decomposition)
Soil properties	abiotic properties of the soil habitat (e.g., water and nutrient contents) number and biomass of species or organism groups	abiotic properties of the soil habitat (e.g., water and nutrient contents) number and biomass of species or organism groups activity of plant residue decomposition activity of organic waste decomposition
Parameters	abundance and biomass (e.g., of earthworms, soil bacteria, and fungi on a species or genus level), diversity indices (e.g., functional diversity)	soil respiration, nitrogen mineralization, microbial biomass, number and activity of specific decomposer organisms
Methods	PCR-DGGE or T-RFLP of total soil 16S-RNA direct count, selective plating, etc. phospholipid fatty acids (PLFA) diversity within key groups (Mycorrhiza, suppressive microorganisms)	CO ₂ production or O ₂ consumption under controlled conditions microbial biomass by fumigation-extraction or substrate-induced respiration methods DGGE or TRFLP of 16S-RNA of decomposer communities enzyme patterns decomposition in litterbags
Indicators	different diversity indices	basal respiration, metabolic quotient, ammonification potential, etc.

20.2.2.3 Soil Properties and Analytical Parameters

Soil functions are, generally, processes or potentialities depending on soil properties. Functions frequently are more defined in a conceptual manner and cannot directly be quantified. Thus, for assessing soil quality, normally not the functions but the properties representing them are measured. Depending on the specific soil quality of interest and the functions linked with it, a selection of soil properties will be done.

Soil properties are described in different degrees of resolution. For example, the property “amount and activity of soil organisms” is a superordinate property, which itself cannot directly be determined. It can gradually be subdivided into the properties “quantity,” “diversity,” and “activity” of soil organisms. These are not yet measurable but can again be subdivided into directly measurable parameters (e.g., soil microbial biomass or earthworm biomass) (table 20.1). Finally, there often exist several estimation methods for each parameter, which again could have an effect on the result. A method selection will be necessary to reach the desired precision with appropriate costs. Whereas for theoretical considerations normally often superordinate properties are used, measurable parameters are necessary for the concrete assessment of soil quality.

As not all relevant soil parameters can be assessed, indicator parameters are selected. For these parameters additional requirements have to be taken into account (OECD 1997).

Table 20.1 gives an overview of soil properties, parameters, methods, and indicators related to the biotic soil functions “diversity of soil organisms” and functions based on biological activity.

Additionally, methods used for soil quality assessment and especially in long-term field observation have to fulfill several conditions (see section 20.5.1).

With respect to soil biological diversity different aspects have to be derived from these considerations:

- Biological diversity may be of interest as a result of the habitat function or as a basis for other soil functions (e.g., for organic matter decomposition). Depending on the function to be considered, different parameters have to be assessed.
- With respect to the habitat function, soil biodiversity is the endpoint of this function. Factors determining the habitat function are abiotic (e.g., water, air, and chemistry of the soil). A “good” soil quality may be indicated by a favorable abiotic state of the soil (e.g., abiotic parameters are in an optimal range of values).
- Concerning other soil functions based on biological activity, endpoints could be process rates or mass parameters. Biodiversity possibly is a factor but does only partially contribute to a given soil function, together with, for example, abiotic soil parameters, the presence of plants, or climatic conditions. Diversity of those organisms should be preferred that are involved in the processes underlying the soil function to be considered.

The following methods for assessing microbial community structures are presently in use: analyses of total DNA by different fingerprinting techniques (RFLP, DGGE, or SSCP), fractionation and analyses of fatty acids (e.g., PLFAs) or substrate utilization patterns (Degens and Harris 1997; Widmer and Oberholzer 2003). However, all these methods fail to fulfill basic requirements for biodiversity estimations, because their taxonomic resolution is limited. The detection unit of all methods (peak on a genetic fingerprint, specific fatty acids or the utilization rate of a single carbon source) may be shared by unknown numbers of species. Organisms in these groups do not necessarily derive from a monophylogenetic group nor represent a functional group of species performing the same processes. So these methods offer a certain contribution to diversity but not the entire information that the term “diversity” implicates. It is obvious that the detection of diversity at the species or even at the strain level is not feasible, since detailed knowledge on the organisms that live in soils is still missing. A solution may be to define “operational taxonomic units,” which can be detected in order to assess soil microbial diversities.

20.2.2.4 Relationship between Soil Functions and Soil Properties

Soil functions are related to soil properties in a multilateral way. On one hand, one function can depend on different soil properties. On the other hand, one soil property may influence several soil functions. For an assessment of soil quality and especially for the selection of soil properties as soil quality indicators, the relationship between soil functions and soil properties plays a key role. Even though all soil properties affect in some way all functions, specific relations appear to be more important. Candinas et al. (2002) undertook a rough estimation of how strongly a possible deterioration of a soil property can affect a soil function (table 20.2).

In table 20.2 all soil properties are assigned, which exert a relevant influence on soil functions. From these considerations it follows that rooting depth, structural constitution, and content of organic carbon are those soil properties that exert the potentially most comprehensive influence on the soil functions considered here. They are, thus, very important for sustainable land use. Furthermore, we see that the functions “metabolization and decomposition” and, to a lesser extent, “plant biomass production” are affected by most soil properties. We therefore conclude that these represent key functions. Consequently, properties and the related parameters that indicate the state of these key functions are very important in assessing soil quality. Especially for the function “metabolization and decomposition” the microbiological properties “quantity and diversity” and “activity of the soil organisms” play a preponderating role. The special role and the importance of biological parameters are extensively described in section 20.3.

20.2.2.5 Relationship between Biodiversity and Soil Functions

The relationship between the diversity of soil organisms as a soil property and organism-supported soil functions (e.g., decomposition of organic matter, nutrient cycling, or suppression of soilborne

TABLE 20.2
Relationship Between Soil Functions and Soil Properties

Soil properties	Habitat functions				General ecological functions				“Anthropocentric” ecological functions		Agronomic and hydrologic functions	
	Acquiring space	Water reservoir	Gas exchange	Heat storage	Buffering of the soil reaction	Substance storage	Metabolization/ decomposition	Filtering capacity (physical)	Diversity of plants	Diversity of soil organisms	Production of plant biomass	Water balance
Rooting depth	3d	3d	0	3d	3d	3d	1	3d	0	0	3d	3d
Texture, stone content	i	i	i	d	i	i		i			i	i
Structure	3d	3d	3d	2d	0	1	2i	3d	0	0.5	3d	3d
Stability	2i	2i	2i	1	0	0	1	1	0	0	2i	2i
Quantity and diversity of soil organisms	0	0	0	0	0	1	3d	0	0	3d	0	0
Activity of the soil organisms	0	0	0	0	1	0	3d	0	0	0	2i	0
content and quality of organically bound carbon	1	2i	1	2d	1.5d	2i	1	1	0	2d	2i	2i
soil reaction	0	0	0	0	3d	2i	3i	0	0	2d	2d	0
Storage capacity for substances	0	0	0	0	2d	2d	1	0	0	0	1	0
nutrient content incl. salt content	0	0	0	0	0	0	1	0	3d	0.5	3d	0
Pollutant content	0	0	0	0	0	0	2i	0	2d	1.5d	2d	0

Numbers indicate how strongly the possible impairment of a soil property can affect a soil function. A scale is used from 0 to 3, where 0 means that a substantial change of the soil property has no influence, and 3 means that it has an essential influence on the soil function (i.e., the soil function is completely disrupted). It was differentiated between properties with direct (d) or an indirect (i) influence on soil functions.

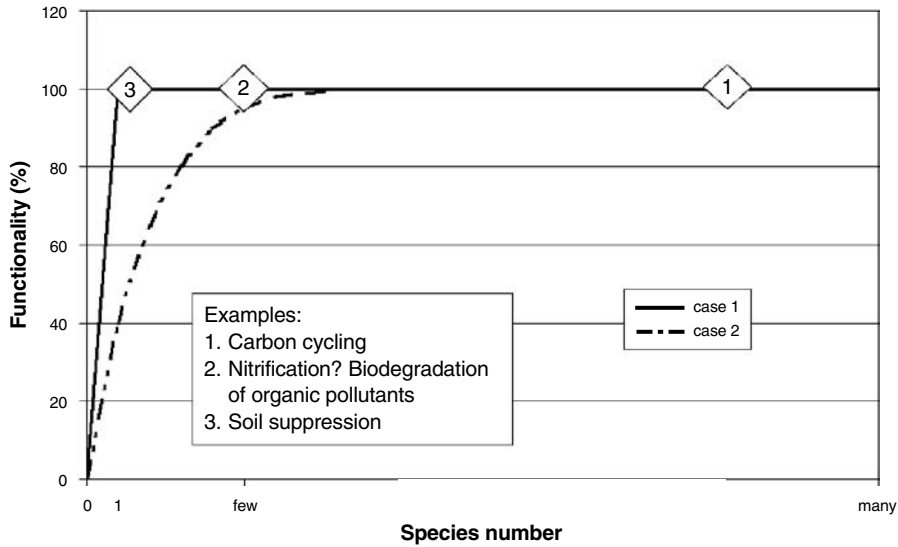


FIGURE 20.1 Schematic relation between species number as indicator for diversity and soil functionality.

plant pathogens) is far from being clear. This is due to the redundancy between soil organisms with respect to soil functions. The more species are involved in a soil function, the higher is the redundancy. Additionally, the relation between species number and function might be different depending on the type of function (Griffiths et al. 2000, 2001).

In figure 20.1 two hypothetical types of relationships (case 1 and 2) are presented. In case 1 one species is the complete carrier of one function. In case 2, functionality progressively decreases as the number of species falls to zero. The following examples should illustrate possible relations between function and species number.

- **High redundancy:** In example 1, which might stand for “carbon cycling,” “decomposition of plant residues,” and “nitrogen mineralization,” a lot of species are involved in this function and a reduction by up to 50% would not affect the functionality at all (fig. 20.1). This relationship has been demonstrated in two destructive studies, where soil biodiversity has been reduced by chloroform fumigation (Griffiths et al. 2000) or reintroduction of diluted cell suspension to sterilized soil (Griffiths et al. 2001). Although overall biodiversity had been reduced by up to 50% general processes like incorporation of thymidine (indicator of DNA synthesis) and leucine (protein synthesis), substrate-induced respiration, microbial growth rate after substrate addition, and decomposition rate of plant residues were not affected. It may be argued that below a critical value of species richness to be defined there might be a relationship between diversity and function. Nevertheless, the authors state that they did already use harsh methods to reduce biodiversity, which were somewhat unrealistic (Griffiths et al. 2000).
- **Low redundancy:** Example 2, which may stand for nitrification or metabolic biodegradation of pollutants, both processes where few organisms are involved, shows case-dependent effects of a reduction of diversity. In case 1 only a reduction to 0 species will affect functionality, whereas in case 2 already a reduction from 5 to 4 species would probably result in a slight reduction in function (e.g., nitrification rate). The latter case was equally shown by the destructive study using chloroform fumigation to reduce biodiversity mentioned above (Griffiths et al. 2000). A net reduction of overall biodiversity by up to 50% led to a strong reduction in nitrification and methane oxidation.

Nevertheless, in the destructive study using the dilution technique nitrification was not affected by the reduction of biodiversity (Griffiths et al. 2001).

- No redundancy: Example 3 illustrates functions based on only one organism (e.g., specific cases of soil disease suppression or the role of the deep dwelling earthworm *Lumbricus terrestris* in the formation of deep continuous soil pores and its importance for water infiltration in Mid-European fine-textured soils). In this case the species number (here: 1) is completely linked to the function.

There will be a lot of intermediate cases between the cited examples. An additional aspect, besides the quantitative realization of a function, is the security that a special function will stay active. The more organisms are involved in a given function the higher is the probability that enough of them will survive after an impact on the soil to ensure the function. The resistance of the soil (function) against an impact is high. Also, the regeneration potential of the soil (function), its resilience, might be higher if it is assumed a more redundant soil microflora and fauna.

The relationship between biodiversity and resistance or resilience was also studied by Griffiths et al. (2000, 2001) in the two above-cited destructive experiments. The decomposition process was more resistant to persistent stress (CuSO_4 addition) in soils with a high biodiversity than in soils with a low biodiversity. Although resilience of this process after persistent stress was low independently from the level of biodiversity, it was higher after transient stress (soil heating) in the soil with higher biodiversity than in the soil with lower biodiversity (Griffiths et al. 2000). Nevertheless, using the dilution approach, no relation between biodiversity and resistance or resilience of the decomposition process to transient or persistent stress was observed (Griffiths et al. 2001).

20.2.3 ASPECTS OF SOIL QUALITY ASSESSMENT

Because of sometimes different use of terms, our definitions are presented here: Assessment is a process of collecting information (e.g., measurement of soil parameters) and the classification of the results by comparison with reference values. Evaluation is the following interpretation of possible differences and a value judgment.

For soil quality assessment specific emphasis has to be put on the periodicity and date of observation (cf. section 20.2.3.1) and on the choice of an adequate reference system (cf. section 20.2.3.2), besides standardization of the methods. Finally, often several and sometimes conflicting land uses have to be considered. In this case different soil qualities have to be assessed and balanced (cf. section 20.2.3.3).

20.2.3.1 Periodicity and Date of Observation

Because soil quality-related soil properties and, especially, biological properties fluctuate in function of climatic conditions and agricultural practices within a year, specific attention has to be paid to the period or date of observation (e.g., Rogers and Tate 2001).

Joergensen et al. (1994) showed for soils in the temperate zone that the microbial biomass follows a time course during the year with low values in winter and maximum values at harvest. Short time variations and sometimes typical time courses also occur for many other soil properties during the vegetation period, during a whole year, or even during a crop rotation. Besides biological properties this happens also, for example, for physical (bulk density, porosity) and chemical properties (nutrient contents or pH value). For the estimation of biological soil parameters Schinner et al. (1996) recommend early spring as an optimal sampling date. This date might also be the best suited for molecular analysis of the population of soil organisms (Hassink et al. 1991). Nevertheless, with probably higher organism numbers in summer, more species might be detectable above the detection limit, giving a more adequate picture of the real species number. Also, zoological investigations mostly should be performed in summer, as many species may not be detected in early

spring because of low abundance or activity. The optimum timing of sampling has to be tested before starting a monitoring program.

Also, soil parameters might be affected by severely short time impacts. So, number, amount, and diversity of earthworms is drastically reduced by plowing and recover mostly within the following 1 or 2 years (Wyss and Glasstetter 1992; Buckerfield and Wiseman 1997). Therefore, short time changes of single soil properties do not necessarily mean a significant reduction of soil quality. Soil quality changes should be observed and assessed in a middle time scale. The time scale is depending on the agricultural system and therefore the observation period should be at least one or two crop rotations. The duration and the scale of reestablishment of the original state after a short-term impact may be considered as an indicator of the resilience of a soil.

Soil quality assessment should be done within a reproducible and most stable situation within the crop rotation and within the year. An optimal and adequate sampling strategy should be developed and used.

20.2.3.2 Choice of a Reference with Respect to Land-Use Systems and Climatic Conditions

The soil quality in different land-use systems may depend on the same functions but is assessed using different scales.

The assessment scheme for each soil property depends on the status of the site (type of ecosystem and land use) (e.g., intensively used arable land, intensively or extensively used or natural grassland, etc.), and should include soil and land-use-specific schemes. As shown in figure 20.2, the microbial biomass on a conversion land (e.g., conversion of grassland to arable land) may be assessed using two different reference systems. The microbial biomass in the example is 25% lower than in a reference grassland soil. On the contrary, using arable land as a reference, the microbial biomass is about 50% higher than the reference arable soils. If the soil, now arable land, is assessed without knowing the land-use history, high values are found as compared to “old” arable soils. From the ecological point of view comparing this conversion soil with arable soils as reference is justified and necessary for the following reasons: low values are typical for arable soils and can hardly be increased by agricultural management. Thus, high microbial activity has to be considered

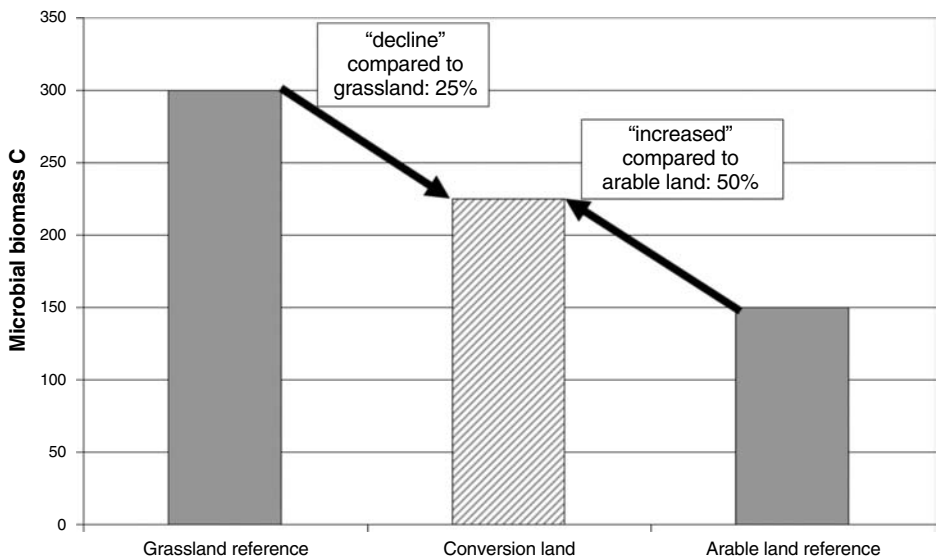


FIGURE 20.2 Assessment of microbial biomass values on conversion land leads to different results depending on the reference (e.g., grassland or arable land).

as an ecological risk due to possible nitrogen leaching, especially during periods without nutrient-demanding plants.

As primary production and organic matter decomposition depend on climatic parameters (i.e., temperature and soil water) regional aspects have to be taken into consideration for assessment schemes of soil quality (e.g., Wardle 1992; Oberholzer and Höper 2000). Nevertheless, climatic parameters are less strongly correlated with biological parameters than abiotic soil parameters. This is also due to the fact that some soil parameters (e.g., soil pH and organic matter content) are more influenced by long-term climatic conditions than by short-term variation.

20.2.3.3 Soil Quality Assessment under Conflicting Land Use

As defined in section 20.2.2.1, soil quality refers to an actual or intended land use and land use is defined in a broad sense. Thus, sometimes several and conflicting soil qualities have to be assessed. For example, if we intensify production from extensive grassland to intensive grassland, nutrient content probably will increase. As a consequence biological activity and carbon content may also increase. If this site is intended as productive grassland, then these increasing soil properties show an improvement of soil quality. On the other hand, diversity of soil organisms and probably diversity of plants on this site might decrease, which indicates a clear deterioration of the habitat function of the soil. In this case it is not a matter of soil quality assessment to weight these two converse effects, but a decision based on the conflicting interests of land use.

With respect to soil quality assessment based on the production function of the soil, the following principle is valid: the higher is soil biological activity, the better is the soil quality. However, when the water balance function and the filtering capacity of the soil *are* more important (e.g., in drinking water catchment areas), soil quality has also to be defined with respect to groundwater contamination and soil properties have to be assessed and evaluated differently. In figure 20.3 soil qualities for plant production and for groundwater quality are schematically presented as a function of the total nitrogen

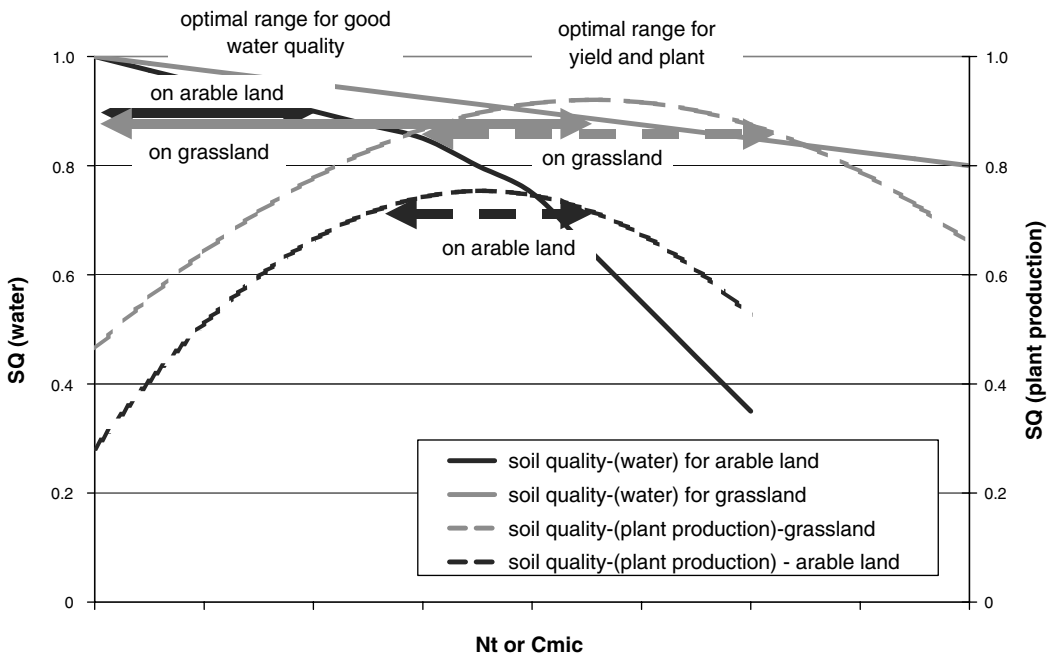


FIGURE 20.3 Hypothetical relation between total nitrogen (N_t) or soil microbial biomass (C_{mic}) and soil quality (SQ) with respect to groundwater quality (water, continuous lines) or plant production (broken lines) for arable land (black) or grassland (grey). Arrows indicate optimal ranges of the soil parameters.

(N_t) or microbial biomass (C_{mic}) for arable and grassland soils. Optimal ranges for N_t and C_{mic} are indicated. From this hypothetical example it can be concluded that under grassland use, there can be defined an optimal range of N_t and C_{mic} for both soil qualities (e.g., the optimal value range for both soil qualities overlay at mean concentrations). On arable land this might not be possible and a decision has to be made upon which system, water caption, or plant production, has to be favored.

20.3 IMPORTANCE OF BIOLOGICAL PROPERTIES IN SOIL QUALITY ASSESSMENT

20.3.1 DETECTION OF EFFECTS OF CHEMICAL AND PHYSICAL CHANGES ON SOIL ORGANISMS

A special role in the assessment of soil quality belongs to the biological soil properties. Assessing chemical properties is suitable to detect chemical impacts and assessing physical properties is suitable to detect physical impacts. Thus, both types of methods are adequate to detect the corresponding changes in soil properties. Biological soil properties (e.g., quantity, activities, and composition of the soil micro flora) are predominantly conditioned by their chemical and physical environment including toxic effect of pollutants. Consequently, the microbiological properties of a soil are the direct result of chemical, physical, and biological properties over a certain time. Thus microbiological parameters of the soil represent indicators of the soil quality, which can indicate in an integrating way short-, middle-, and long-term changes of soil quality. Additionally, biological analysis can detect effects, which cannot be detected using only chemical or physical analyses. So biological procedures

- show the biological effect of pollutants at the effect place (e.g., bioavailability and mobility of the pollutants),
- seize the effect of toxic materials and their metabolites that in the course of control analytics would not be analyzed, and
- integrate synergistic effects of different load types (e.g., chemical and not chemical).

Additionally, soil microorganisms are not only affected by chemical and physical effects, but by their decomposing activity the microorganisms also affect the physical and chemical soil properties (e.g., aggregate stability and pH value), and, thereby, soil fertility.

Biological soil properties comprehend quantity, diversity, and activity of soil organisms. While, for example, nutrient content is a very clear description of a property, a specific quantity or activity of soil microorganisms normally is a rather variable property. The result of a microbial biomass does not indicate of which species it consists. Therefore these parameters are also called summarized (global) parameters. The knowledge of the composition of the population for a specific biomass or activity would be very useful for the interpretation of results, especially in situations where diversity could be reduced and possibly some functions are solely realized by few species (cf. section 20.2.2.4). This not only by the fact that already by existing laws a high suitable soil diversity itself is demanded but, moreover, because further damage of the concerning species would lead to endangerment of this function.

Biological parameters show effects of chemical and physical influences and their interactions over time. Effects of these interactions can only be detected using biological methods.

20.3.2 USE OF SOIL BIOLOGICAL PROPERTIES TO DEFINE CHEMICAL AND PHYSICAL THRESHOLD VALUES

Biological soil properties are furthermore important for the evaluation of chemical and physical soil properties.

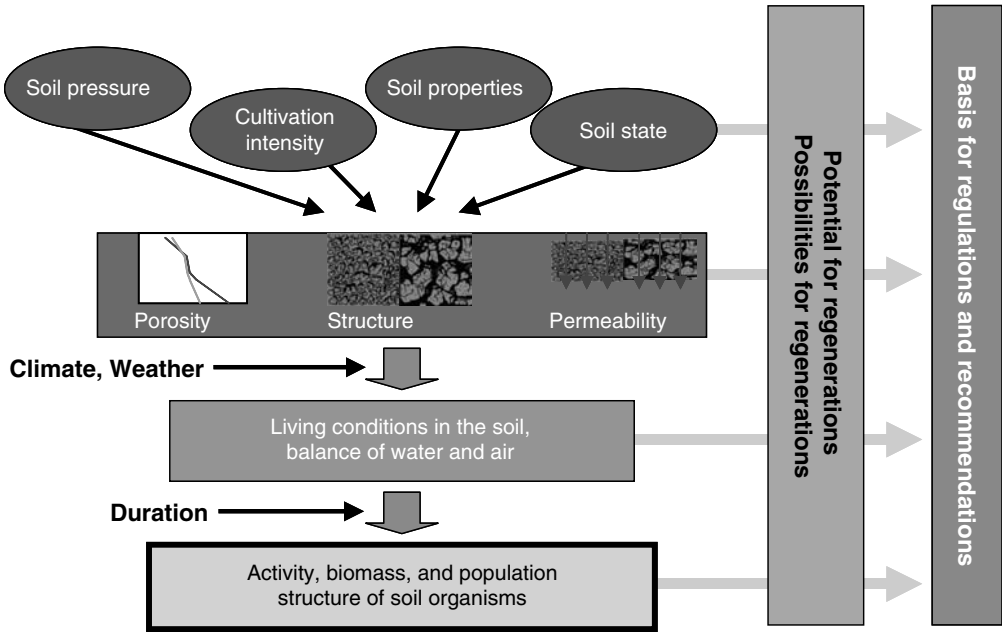


FIGURE 20.4 Schematic relations between soil physical impacts and biological soil properties.

A content of a substance (nutrients or pollutants) in the soil itself cannot be assessed. The criterion for assessment of chemical soil properties is always derived from a function or an influence on one or more functions, especially influences on plants and soil organisms, but also interactions between chemical properties and influences on physical properties. Thus nutrient contents and pH value are normally assessed with a focus on plant growth. Organic matter content is assessed regarding multiple effects on chemical, physical, and biological properties. Threshold values for pollutants as one of the most important criterion in soil conservation are based mainly on their influence on soil organisms and plant growth.

Analogous to chemical soil properties, physical soil properties are also assessed due to their effects on soil functions. Thereby the influence on biological functions is very important. Porosity is the most important property for the functions of balance of air and water on the site-specific scale as well as on the regional scale. Additionally, changes of porosity by soil compaction have to be assessed above all with respect to their influence on life in the soil. Compared to pollutants, for physical properties not only the actual state is important but also the duration of a disturbance and the possibility of regeneration (fig. 20.4). Furthermore, the effect is depending on other site properties such as climatic conditions, especially the amount and the distribution of precipitations. Whereas threshold values for pollutant contents to a great extent already exist, corresponding regulations for physical impacts in most of the countries are missing. Instead of a threshold value for the state of physical properties it is also possible to define threshold values on the level of processes or general behavior patterns for management such as limitations for the weight of agricultural machines or the statement, “Avoid soil compaction and soil erosion by appropriate management practices” (see section 20.2.1).

20.4 ASSESSMENT AND EVALUATION OF BIOLOGICAL PARAMETERS

Assessment and evaluation of microbial parameters for a given site and land use may be done in the following manner:

- Definition of reference situations (e.g., plough layer of arable soils or upper 10 cm of grassland soils) in a given climatic region.
- Derivation of reference values and specific variability of biotic parameters as a function of abiotic, easily available soil properties for each land-use system. This should be done using a representative data set, for example, data from soil monitoring or landscape investigations.
- For assessment measured biotic parameters of a given site are compared to reference values. The results are classified in a range between very low to very high.
- The evaluation then considers not only the values themselves, but takes into account the situation of the site with the appropriate soil quality (fig. 20.2). So in water catchment areas higher values of microbial parameters have to be valued more critically, whereas in areas with low risk of nitrate-leaching values in the upper half of the reference range are appropriate.

The principles of assessment and evaluation under different circumstances are quite clear. The main problem now remaining is the missing or small data basis for the necessary reference values for many regions and land-use systems. Additionally, the knowledge for the interpretations with respect to different soil qualities is still incomplete.

20.4.1 REFERENCE SYSTEMS

To evaluate the effects of land-use practices as well as chemical and physical impacts on soil microorganisms under *in situ* conditions, reference values are needed.

In the following, the establishment of a reference system for microbial biomass is described, a parameter summarizing the mass of all microorganisms in soil. The question often comes up which microbial biomass contents can be considered as normal and to which extent a deviation from this expected value can be accepted. This can only be answered under the following conditions:

- Use of standardized sampling strategies and methodology
- Consideration of spatial and temporal variation
- Consideration of climatic aspects (long-term climate) and variation between regions (for a national system) or between countries (for an international system)
- Consideration of land-use systems

For the establishment of reference value systems or evaluation schemes, different approaches are principally possible. A discussion of them and the development of an approved reference system based on multiple linear regressions are presented in Oberholzer et al. (1999). For the same approach Höper and Kleefisch (2001) used the data of the long-term soil monitoring program of Lower Saxony (section 20.5).

A short description of this monitoring program is given here, an extended description in section 20.5. Until 1999 70 soil-monitoring plots were established in Lower Saxony on sites representative for soil types and regional land-use practices. Each monitoring plot consists of four subplots (area 256 m²) of which independent samples were taken in order to integrate spatial variation on this scale. In the first year, these samples were analyzed for soil texture, organic carbon, total nitrogen, and soil pH(CaCl₂). For microbial biomass (substrate-induced respiration [SIR]; Anderson and Domsch 1978; Heinemeyer et al. 1989), additionally, interannual variation was included: soils were sampled over a period of 4 years, once every year in early spring (mid-February to the end of March).

Multiple linear regressions were calculated between soil microbial biomass C contents and abiotic soil properties. Due to lognormal distribution, microbial biomass and some of the abiotic soil characteristics were log transformed (e.g., organic carbon, total nitrogen, and clay content). For grassland soils, the upper depth of the sampled layer (0 for the layer 0–0.1 m and 0.1 for the

layer 0.1–0.2 m) was also included in the regression equation as a parameter. pH(CaCl₂) and organic matter content were considered to be constant during this time period of 4 years. Polluted soils were excluded from the calculation.

The following regression equations and standard errors were obtained for arable and grassland soils (table 20.3). These multiple linear regression equations can be used for the evaluation of the microbial status of soils by comparing the predicted and measured values for microbial biomass. Sampling and measurement has to be done under the above-described conditions.

TABLE 20.3
Results of the Multiple Linear Regression (MLR) Calculations for Microbial Biomass (C_{mic}) in Arable and Grassland Soils

	Number of sites	n	MLR	Standard error (log10)
Arable (0–0.2 m)	46	961	$\log_{10} C_{mic} = 2.45 - 0.0032 \text{ sand [\%]} + 0.41 \log_{10} Nt [\%] + 0.06 \text{ pH}_{CaCl_2} [-]$ ($R^2 = 0.738$)	0.1189
Grassland (0–0.1 m; 0.1–0.2 m)	11	334	$\log_{10} C_{mic} = 0.48 \log_{10} Nt [\%] + 0.39 \log_{10} \text{clay [\%]} - 2.62 \text{ depth [m]} + 0.10 \text{ pH}_{CaCl_2} [-] + 0.29 \log_{10} C_{org} [\%]$ ($R^2 = 0.913$)	0.0999

Depth = upper depth of sampling layer (e.g., 0 m for sampling layer 0–0.1 m); Nt = total nitrogen; C_{org} = organic carbon

Results for arable soils from Höper and Kleefisch (2001).

A deviation between the predicted and the measured values can be evaluated as following. Predicted value ± 1 standard error (of the multiple regression models) should be regarded as normal. Predicted value $\pm >1 - 2$ standard errors are proposed to be interpreted as high or low and if the measured value deviates more than 2 standard errors from the predicted value, the measured values are considered as very high or very low (table 20.4).

TABLE 20.4
Evaluation of Measured Microbial Biomass in Arable and Grassland Soils

Measured value related to predicted value %	Arable soils		Grassland soils	
	min	max	min	max
Very low		<57.8		<63.1
Low	57.8	<76.1	63.1	<79.5
Normal	76.1	131.5	79.5	125.9
High	>131.5	172.9	>125.9	158.4
Very high	>172.9		>158.4	

Note: Limits of the quotient of measured values to values predicted from table 20.3.

A similar reference system has also been established on a binational basis, derived from Lower Saxony and Swiss microbial soil-monitoring data (Oberholzer and Höper 2001). In this context, also climatic data (i.e., mean annual precipitation) had a certain but small influence on the prediction of microbial biomass. Nevertheless, the standard error was slightly higher than in the regional reference system for each regional system alone (standard error of 0.123 and 0.119, respectively).

20.4.2 EXAMPLES FOR ASSESSMENT AND EVALUATION

20.4.2.1 Assessment of Polluted Soils

For the assessment of possible adverse effects of polluted soils on soil organisms, frequently ecotoxicological tests are used. Mostly, these tests work with non-autochthonous soil organisms testing short-term effects. If a reference system is available, an assessment of ecotoxic effects on the in situ microflora is possible by comparing measured values with the reference values (e.g., derived following table 20.3). An example is shown here for a 2,4,6-trinitrotoluene (TNT)-polluted site (Frische and Höper 2003). Soil from several plots following a gradient of TNT concentration was analyzed for soil microbial biomass at the beginning of and during a bioremediation field trial. In parallel, microbial biomass values were estimated from sand and total nitrogen contents as well as pH(CaCl₂) using the equation for arable land in table 20.3. The measured values were assessed using the scheme in table 20.4. The low and nonpolluted plots showed normal to very high soil microbial biomass values (fig. 20.5). At the very high concentration level of more than 1000 mg TNT kg⁻¹ soil a very low microbial biomass was detected, corresponding to 34 and 16% of the predicted values.

A second example is cited from the Lower Saxony Soil Monitoring Program (Höper und Kleefisch 2001). The question was whether a heavy metal-, dioxin-, and furan- polluted Fluvisol used for grassland in the flood plain of the Elbe River showed a noticeable deviation in microbial biomass compared to unpolluted soils. The fluvisol contained tetrachlorodibenzodioxines (TCDD, 318 ng kg⁻¹ soil) and tetrachlorodibenzofuranes (TCDF 2470 ng kg⁻¹ soil) in the upper 5 cm of the soil, and heavy metals, particularly mercury (19,9 mg kg⁻¹ soil), cadmium (14,3 mg kg⁻¹ soil), and zinc (1700 mg kg⁻¹ soil) expressed as total concentrations in 5–10 cm soil depth. Microbial biomass was measured on soil sampled once a year in early spring during 4 successive years on four subplots and assessed using the reference equation for grassland in table 20.3 and the ranges in table 20.4 (fig. 20.6). In almost all situations the measured microbial biomass values were assessed

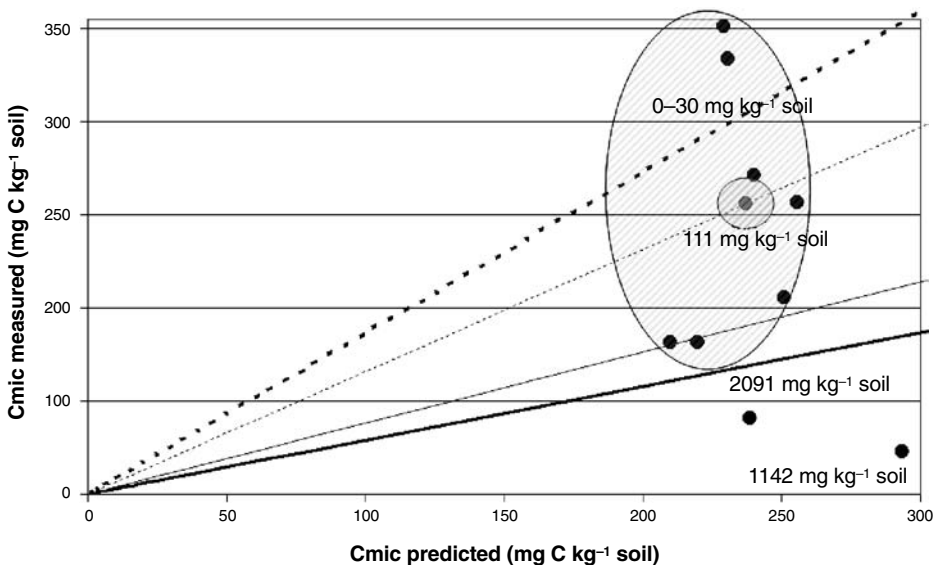


FIGURE 20.5 Predicted and observed values for microbial biomass in 2,4,6-trinitrotoluene (TNT) polluted soils. Measured microbial biomass values in non-, low- (< 30 mg TNT kg⁻¹ soil, light grey ellipse), and medium-polluted plots (111 mg TNT kg⁻¹ soil, dark grey ellipse) are in the range of normal, high, or very high values (table 20.4). Data calculated from Frische and Höper (2003).

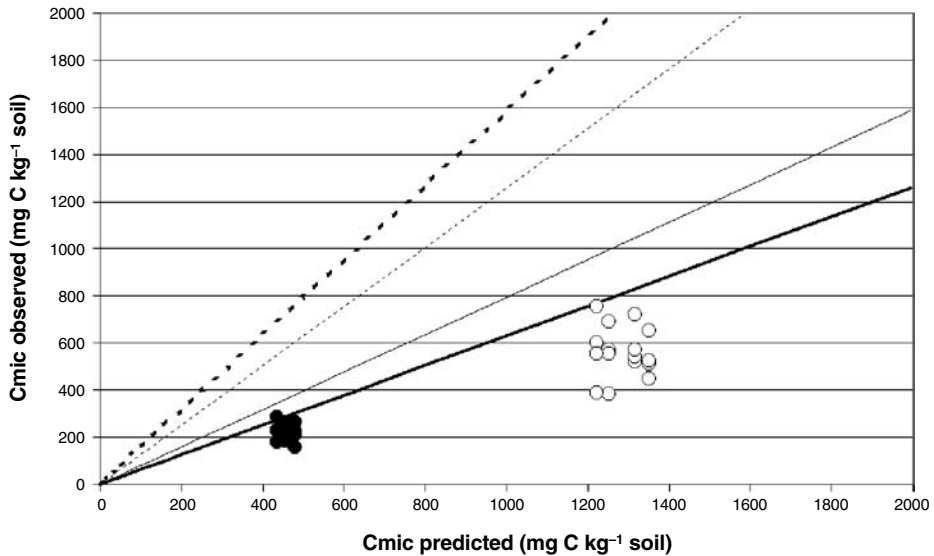


FIGURE 20.6 Predicted and observed values for microbial biomass of a polluted Fluvisol in the layer 0–10 cm (open circles) and 10–20 cm (closed circles). Individual values are shown for each of four subplots and for each of the 4 years.

as being “very low.” Nevertheless, in this case it cannot be ruled out that the regular flooding in winter does also contribute to the low microbial colonization.

By the use of an external reference instead of a control soil, which sometimes is difficult to define and to find, a general assessment of the polluted soils is possible including the information on natural (temporal and spatial) variation to be expected between unpolluted soils of similar characteristics.

20.4.2.2 Assessment of Land-Use System

Assessment of organic farming versus conventional farming. In field trials organic farming has been shown to lead to increasing microbial biomass in soils (e.g., Mäder et al. 1995). In the soil monitoring program of Lower Saxony five fields cultivated by organic farmers during periods of different length are included. The question was whether under these practical conditions (fields are cultivated by the farmers themselves) organically farmed soils contained significantly more microbial biomass than reference (conventionally) cultivated fields and whether the duration of organic farming did accentuate this difference. Microbial biomass was analyzed on soils sampled in early spring in one year on four subplots. The measured results were compared to predicted results using the above-mentioned reference system for arable soils. In almost all situations the measured values for microbial biomass fell into the range of normal values, and sometimes even low values were observed (fig. 20.7). Even if the values of four of the five fields (all except B050) tend to have slightly higher values than expected (10–30% above predicted) it has to be noticed that this deviation falls into the range of spatial and temporal variation of conventionally managed arable soils within the soil monitoring program. Similar results (i.e., no important differences between organically or conventionally managed) were reported by Emmerling (2005) and Oberholzer and Mäder (2003).

Assessment of fallow land. A final example is the evaluation of the effects of fallow programs (e.g., within the Agrarian Politics of the European Union) on soil microbial parameters. Due to the omission of ploughing and the reduction of biomass production of the plants, the humus profile with depth of the soil is changing, which leads to changes in microbial parameters. An additional question is whether the omission of agricultural measures (e.g., ploughing, fertilization, and use of

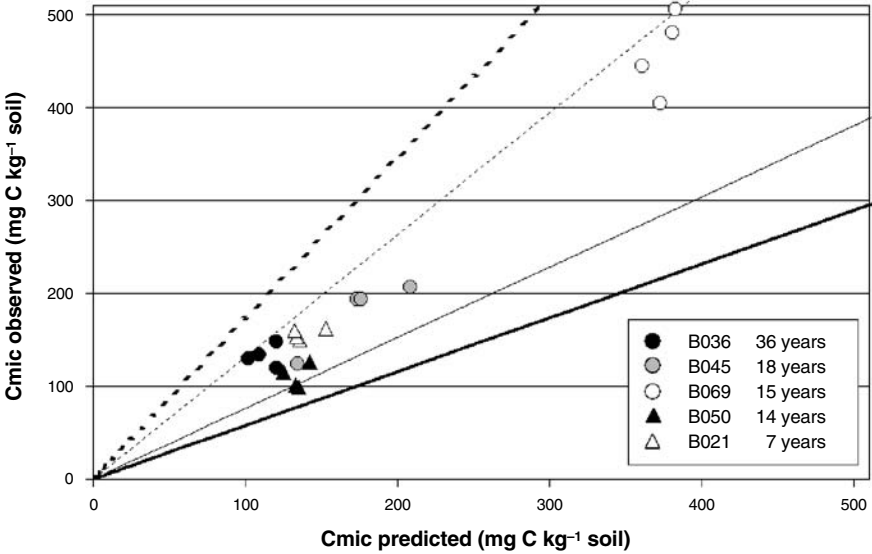


FIGURE 20.7 Predicted and in 2002 measured values for microbial biomass of organically farmed soils (B036 organically farmed since 1966, B045 since 1984, B050 since 1988, B069 since 1987, and B021 since 1995).

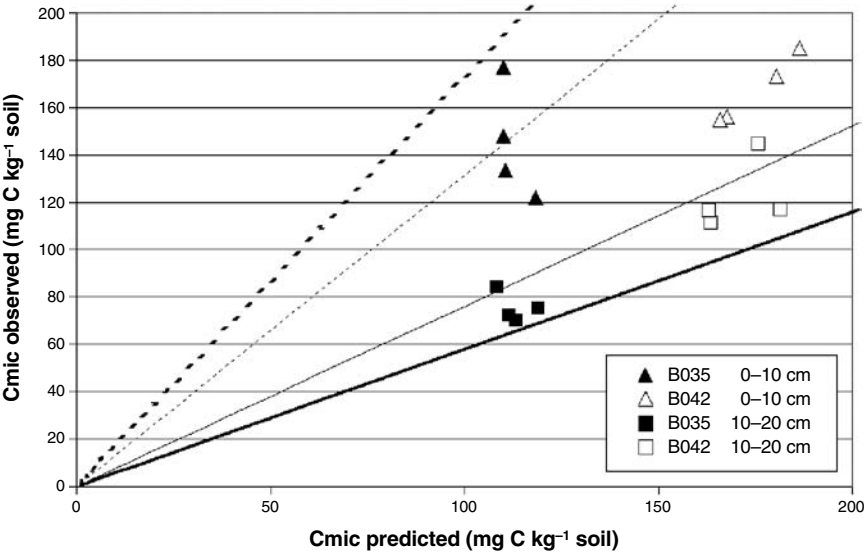


FIGURE 20.8 Predicted and in 2002 measured values for microbial biomass on green fallow soils in the layers 0–10 and 10–20 cm (B035 fallow since 1997, B042 fallow since 1988).

pesticides under fallow land) leads to a “recovery” of biological soil parameters as compared to cultivated land.

Microbial biomass was analyzed on soil material sampled in early spring from two fallow sites. Sampling was performed on four subplots and two layers. Measured values were compared to predicted values and assessed as described above. In all cases soil microbial biomass was clearly higher in the upper layer (triangles) than in the lower layer (squares, fig. 20.8). Whereas microbial biomass was “normal” or, in two cases, “high” in the upper 10 cm, it was “low” in the lower 10 cm. Thus, there was no sign of a general biomass “recovery” (i.e., an increase in microbial biomass

contents) in the upper soil layer, once intensive agricultural practices are stopped including nutrient and pesticide applications. The low contents in the lower layer are due to the reduced carbon input after the omission of ploughing.

20.5 LONG-TERM SOIL MONITORING

20.5.1 CRITERIA FOR THE CHOICE OF PARAMETERS

Due to their indicator function, namely also for changes of abiotic soil properties, microbial parameters are, in general, well suited for long-term soil monitoring purposes. Nevertheless, not each microbial parameter is suitable, and, not only for financial reasons, choices have to be made based on objective criteria (table 20.5).

The costs (criterion f) play an outstanding role in indicator selection, as “hard” abiotic soil properties (e.g., total heavy metal, organic pollutant, and nutrient contents) are often considered as indispensable parameters to be monitored, whereas “soft” biological properties are regarded as additional information. Thus, the complete list of a minimal data set on soil biological parameters as established in preparation of a Danish Monitoring Program (Nielsen and Winding 2002) containing 14 biological parameters might not become reality. Nevertheless, the use of few microbial indicator methods may strongly reduce the costs by reducing the need for an extensive physical and chemical analyses program.

Biological soil monitoring should start with few but well standardized methods (criterion e). Even though standardized, these methods have to be tested for their suitability in long-term soil monitoring.

Specific requirements for biological methods to be used in soil monitoring have to be respected.

- The biotic parameters to be measured should not be too sensitive to short-term climate and weather variations but sufficiently sensitive to changes in management and chemical and physical impacts.
- In order to monitor long-term changes, it is preferable to assess bulk soil instead of rhizosphere parameters and to exclude the impact of the living plant and the incorporation of fresh plant material as far as possible, using appropriate sampling and pretreatment strategies.
- The reproducibility over years if not decades should be assured. This demands a specific degree of standardization, including the whole process from sampling over sample treatment including storage, measurement, and data analysis procedures, as well as method documentation.
- Additionally, the spatial variation should be taken into consideration.

TABLE 20.5
Criteria for the Selection of Indicator Parameters

- a. policy relevance and utility for users
- b. scientific validity
- c. linked to or correlated with ecosystem processes (i.e., ecological significance)
- d. analytical soundness/reproducibility
- e. suitable for routine testing/measurability
- f. cost effectiveness
- g. responsive to variations in management and climate at an appropriate time scale
- h. extrapolation possibilities

Derived from OECD (1993), Doran and Safley (1997), Torstensson (1997), Vos et al. (2000)

Soil microbial biomass and basal soil respiration are the most important soil parameters and have proven to be robust and powerful in soil quality monitoring (Höper und Kleefisch 2001). However, they do not reflect diversities and structures of soil microbial communities. More recently developed methods may serve this purpose. Analyses of community-level substrate utilization, community-level fatty acid profiles, or community-level genetic profiles represent the most commonly used fingerprinting approaches for microbial community structures. However, these methods do not yet meet most of the criteria for robust and applicable indicators and have still limited power for determination of microbial diversity in soil (Widmer and Oberholzer 2003). Thus, further development of fingerprinting indicators is required. We propose to use for long-term monitoring purposes only established and robust soil biological indicators meeting the requirements of table 20.5. Focused research needs to be initiated to establish defined indicators of changes in soil microbial community structures. These indicators need to be included in the set of currently existing indicators in order to allow monitoring of soil biological characteristics at a higher resolution.

Considering the spatial variation, the point-to-point variation should not be overemphasized. In Lower Saxony each monitoring site consists of four subplots, each of 256 m², of which a mixed sample of 16 cores of 4 cm in diameter is taken (Höper and Kleefisch 2001). Under these conditions the spatial variation was moderate. In more than 80% of the cases (combination of year and monitoring plot) the spatial variation coefficient between the four subplots was below 15% and only in less than 3% a variation coefficient of more than 25% was observed (fig. 20.9). Nevertheless, for new biological methods (e.g., molecular diversity assessments), spatial variation should be tested, especially regarding the fact that frequently less than 1 g of soil is analyzed.

Also the temporal variation (e.g., the interannual variation of samples of the same period within a year from the same plot) has to be kept in mind. In the soil monitoring program of Lower Saxony the coefficients of the temporal variation were only slightly higher than the coefficients for the spatial variation between subplots (fig. 20.10). This confirms the high reproducibility of the sampling, storage, pretreatment, and analytical procedures. Only under such conditions trends in microbial parameters can be detected.

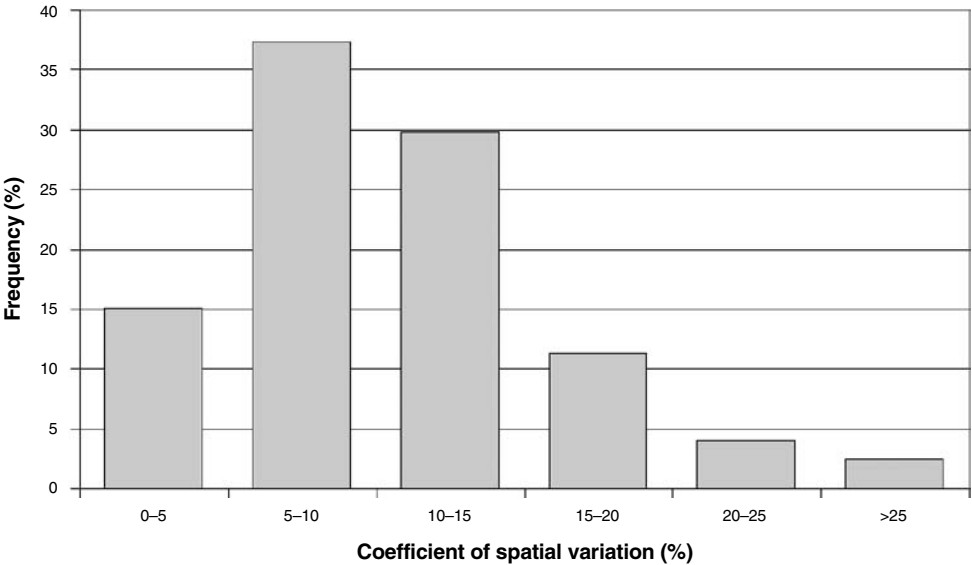


FIGURE 20.9 Frequency of coefficients of spatial variation for microbial biomass in arable soils between four subplots of 256 m² in the years 1996–1999 of the Lower Saxony Soil Monitoring Program (Höper and Kleefisch 2001; modified).

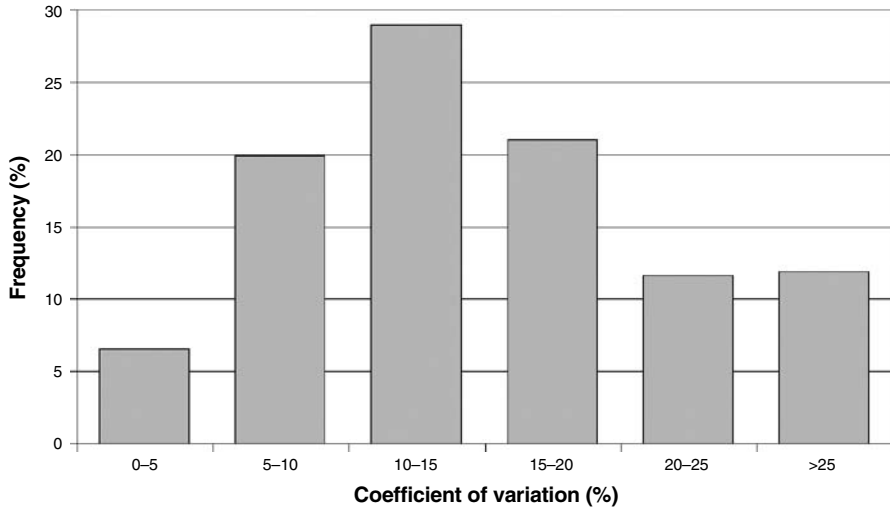


FIGURE 20.10 Frequency of coefficients of temporal variation for microbial biomass in arable soils of the same subplots between the 4 years 1996–1999 in the Lower Saxony Soil Monitoring Program (Höper and Kleefisch 2001; modified).

20.5.2 SOIL MONITORING IN LOWER SAXONY: AN EXAMPLE OF USE

20.5.2.1 Materials and Methods

A general description on installation and operation of soil-monitoring plots in Germany can be found in Barth et al. (2000). Site selection will not be discussed here, as presently a working group is preparing a proposal for a European “soil-monitoring directive” and different criteria and procedures for site selection are discussed.

In the state of Lower Saxony, Northwest Germany, a soil-monitoring program was started in 1991 (Kleefisch and Kues 1997). Within this program soil microbial biomass is measured annually on 49 arable and 18 grassland sites, each with four subplots of 256 m². Soils are sampled in late winter, mid-February, and the end of March and analyzed for microbial biomass using the SIR method and a conversion factor of 30 mg C_{mic} μl⁻¹ CO₂ h⁻¹ (Anderson and Domsch 1978; Heinemeyer et al. 1989; Kaiser et al. 1992).

Within the Lower Saxony Soil Monitoring Program trends for soil microbial biomass were calculated as a linear regression of all four subplots of a monitoring plot over 5 to 7 years. If the regression was significant (t-test, $p < 0.05$) the slope was calculated as mean annual change of the microbial biomass content in the plough layer. If the regression was not significant, the mean change was set at 0.

20.5.2.2 Results and Discussion: Microbial Biomass as Indicator of Soil Acidification

The mean annual change of the microbial biomass was correlated with soil pH (fig. 20.11). At a pH (CaCl₂) below 5.5 no increase of microbial biomass was found and in about 50% of all cases a significant reduction was confirmed. This was essentially true for sandy soils. At a pH above 5.5 in 33% of the cases a significant increase was observed.

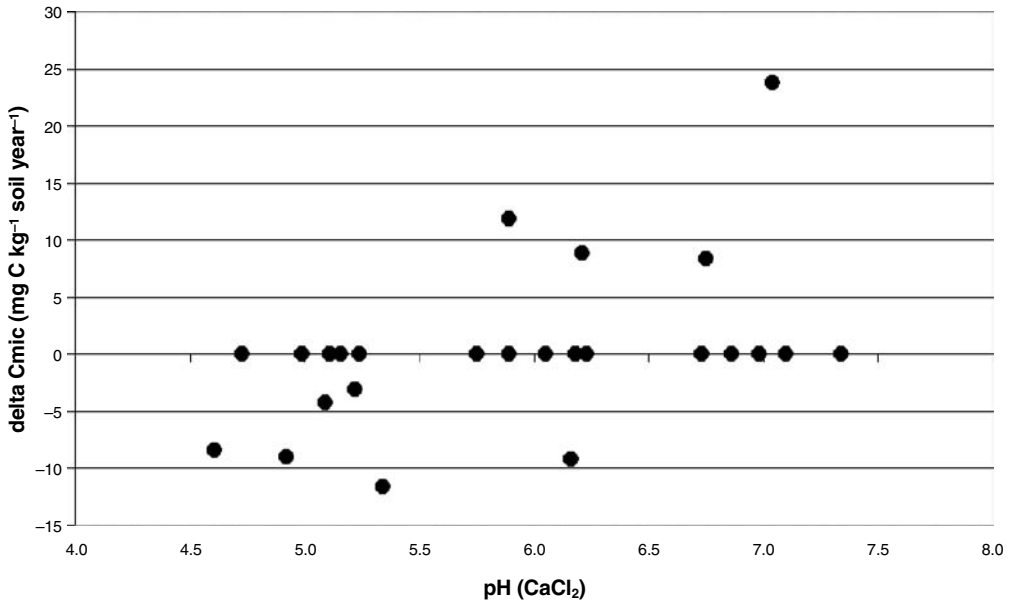


FIGURE 20.11 Trends of microbial biomass over 5–7 years as a function of soil pH. Nonsignificant trends were set at 0 (Overesch et al. 2003; modified).

20.5.2.3 Results and Discussion: Microbial Biomass as an Indicator of Carbon Input

For each year and each monitoring plot the gross annual carbon input was calculated. Inputs of plant and root residue carbon were calculated from the dry matter yields and yield-to-plant residue or yield-to-root residue quotients taking into account the information about the fate of the harvest residues (Höper and Kleefisch 2001). Carbon input by organic fertilizer was calculated from application rates and mean dry matter contents. Carbon input from cover crops, which were only cultivated in a few cases, were calculated from literature data on expected yields and root production. Each carbon input was assigned to the year, where the organic matter was incorporated into the soil by ploughing or other type of tillage mixing at least the upper 10 cm of the soil. The gross carbon input was calculated as a sum of these four carbon sources assuming a carbon content of the dry matter of 50%. The mean gross carbon input was calculated as the mean of one monitoring plot over at least 4 years.

From the relationship of these data to microbial biomass it can be concluded that the trend in microbial biomass depends on the gross carbon input (fig. 20.12). The lower the input the more the biomass content tends to decrease. At a mean annual gross carbon input of less than 5 t ha⁻¹ year⁻¹ only constant and decreasing microbial biomass contents were observed.

The low carbon inputs were more frequently observed on sandy soils. Nevertheless, the relationship between clay content and the yield of a given crop is only very weak, so that the low productivity of sandy soils *per se* was not responsible for the low organic matter input. Moreover, in Lower Saxony, sandy soils are often cultivated with corn for silage. In this case the whole plant is used as animal feed and exported from the field, additionally corn has only a small root system, so that only little root residues remain in the soil. An additional explanation would be a high soil organic matter decomposition due to better aeration in sandy soils.

Nevertheless, soil monitoring is designed to detect changes in soil properties and soil indicators under practical field conditions but is not suited to establish cause-to-effect relationships. Therefore additional evaluation programs of factorized assay are necessary.

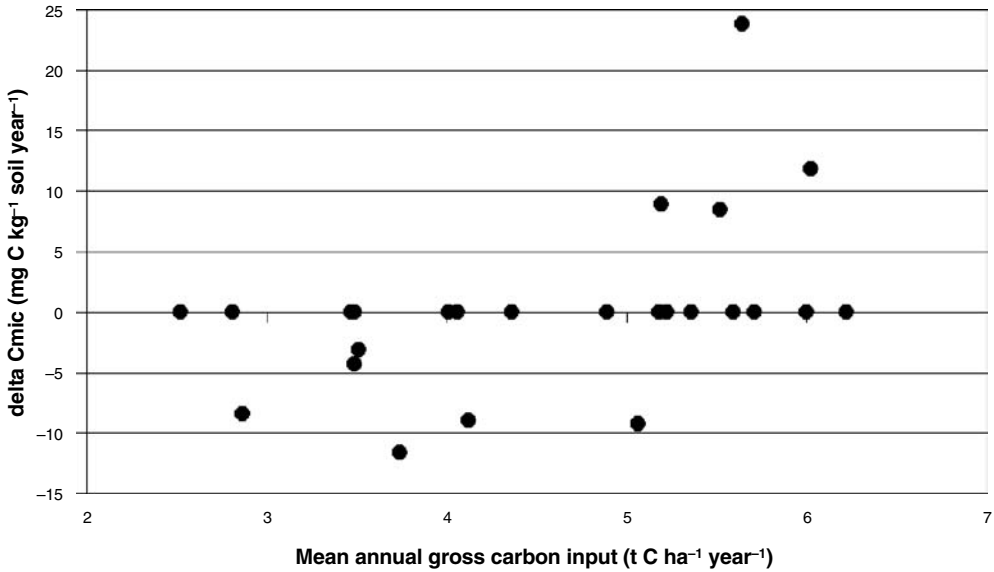


FIGURE 20.12 Trends of microbial biomass over 5–7 years as a function of the mean annual gross carbon input (for calculation, see text; Höper and Kleefisch 2001).

20.5.2.4 Conclusions from Microbial Biomass Measurements in Soil Monitoring for Future use of Diversity Indicators

Microbial biomass as summarizing or “black box” parameter is not suited for the detection of diversity by itself but can serve as an indicator for soil quality and adverse effects on soil micro-organisms. The use of diversity-related methods in soil monitoring is desirable. Nevertheless, the methodological requirements fulfilled by microbial biomass measurements have to be applied to diversity assessments.

The aim of soil-monitoring programs is to detect slow but systematic changes in soil parameters under current land use practices with time, usually over several years or decades. These long-term changes can only be detected after a certain time. The shorter the time, the less the systematic trend is hidden by nonsystematic variations. Therefore, specific attention has to be paid to methodological and parameter inherent variation sources. Besides the analytical method, sampling, storage, and pretreatment also have to be standardized. In a standardized sampling and pretreatment procedure spatial variation between several subplots of some 100 m² (necessary to get representative results from the land-use unit, e.g., a field of several hectare) sampled individually (one mixed sample per subplot) and temporal variation between the same seasons of different years should be significantly below 25%.

20.6 CONCLUSIONS

Soil organisms are under protection of the UNEP Convention on Biological Diversity as well as the Swiss and German soil protection legislation. Following these regulations soil biodiversity has to be protected by itself and as a factor of soil functioning. Moreover, the sustainable use of the biotic resources is the essential part of these rules. Concerning soils, biodiversity does not stand alone, but has to be considered within the concept of soil quality, taking soil functions in relation to actual and intended land use into consideration.

The diversity of soil organisms and, more specifically, of soil microorganisms is a soil property based on the number of species and their abundance. Additionally, considering the activity of

organisms, the functional diversity (e.g., the number and intensity of biological processes in soils) has to be considered. The current methods allow the analysis of parameters of soil biodiversity, which are far away from assessing the diversity in the sense of the UNEP Convention. Determination of phospholipid fatty acids gives a picture of the structure of the soil microflora but cannot be linked to single species. Also, molecular techniques, especially fingerprinting techniques, assess diversity on a genetically based structural level, but not on the species level. Methods on functional diversity consider only a part of biological processes in soils and sometimes only the activity of a part of the soil microflora (e.g., BIOLOG working with cell suspensions). Finally, the relation between biodiversity of species in soil and functional diversity is far from being elucidated. At least, general soil functions in which many microbial species are involved (e.g., plant residue decomposition or nitrogen mineralization) seem to be little affected by a reduction of biodiversity. Only specific microbial processes based on the activity of few species (e.g., nitrification or methane oxidation) are sensitive to species reduction.

Nevertheless, there is a need for long-term monitoring of diversity parameters and their relation to soil functioning. In order to be able to evaluate observations in exact laboratory and field trials, knowledge is required on determinative factors and on temporal and spatial variation on a regional scale. This knowledge can be integrated into a reference system as is shown for the assessment of soil microbial biomass. For the use of diversity methods in long-term soil monitoring, specific requirements have to be fulfilled: high standardization of sampling, storage, pretreatment, and general method, and resulting low temporal and spatial variation. This is indispensable if trends have to be noticed in an early stage in order to be able to counteract.

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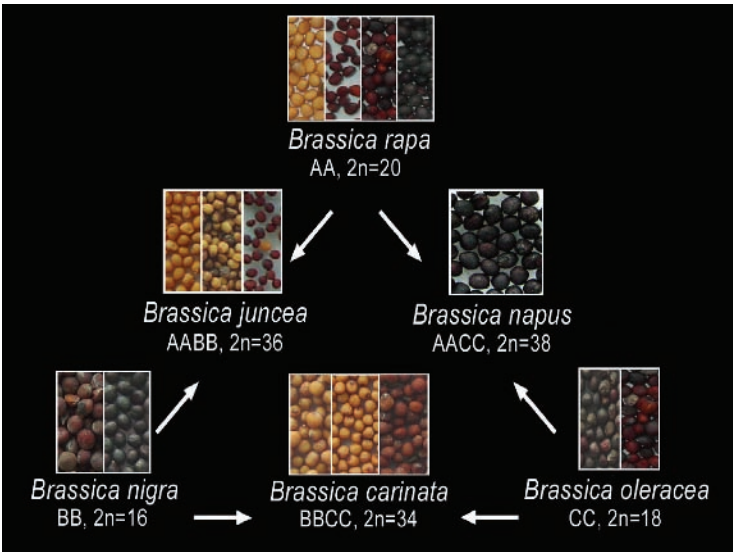


FIGURE 2.1 Scheme showing the origin of the amphidiploid species rapeseed (*B. napus* L.), *B. juncea* and *B. carinata* via natural interspecific hybridization between progenitor *Brassica* species *B. nigra*, *B. rapa*, and *B. oleracea*, respectively. Genetic variation within species is exemplified by different seed colors associated with variable conformation of the seed coat (e.g., fiber content and structure).

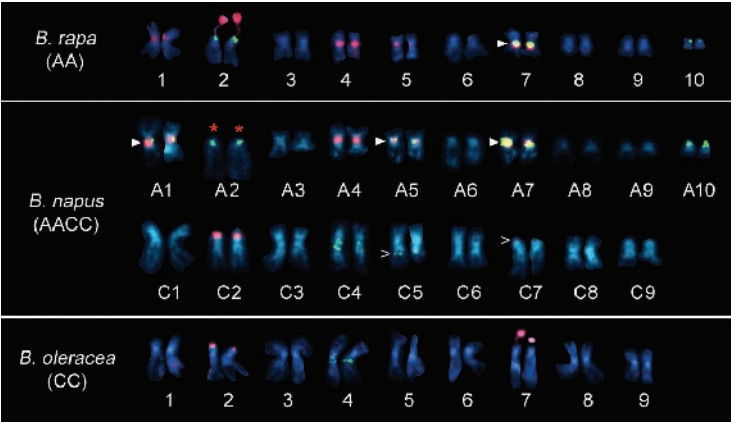


FIGURE 2.2 Karyotypes of rapeseed and its parental species based on fluorescence *in situ* hybridization patterns with 5S (green) and 25S (red) rDNA probes and DAPI staining (blue), for *B. rapa* L., *B. oleracea* L. and their amphidiploid *B. napus* L. [67].

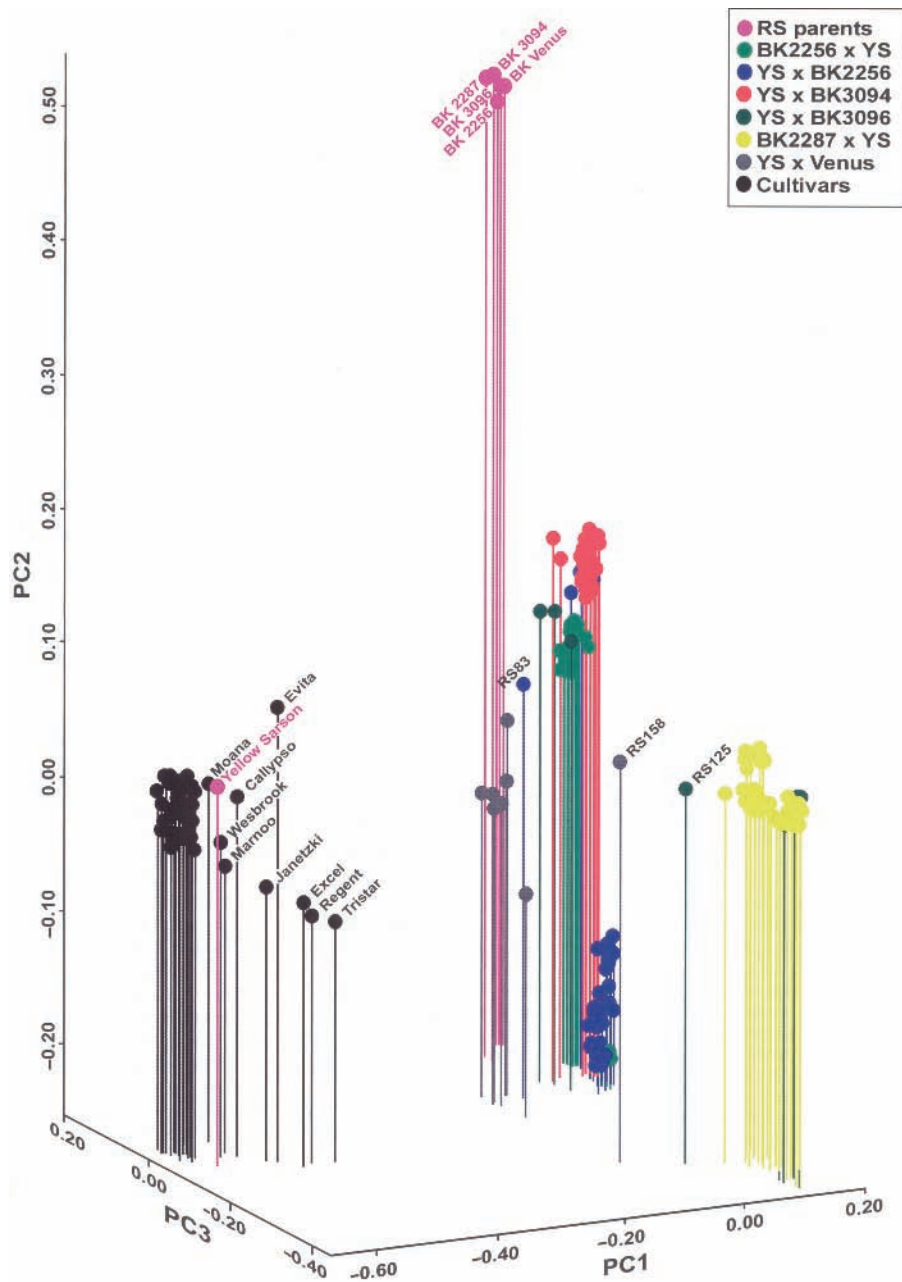


FIGURE 2.3 PCO analysis showing genetic relatedness among a set of spring rapeseed cultivars (black) in comparison with families of resynthesized rapeseed lines (colors) [98].

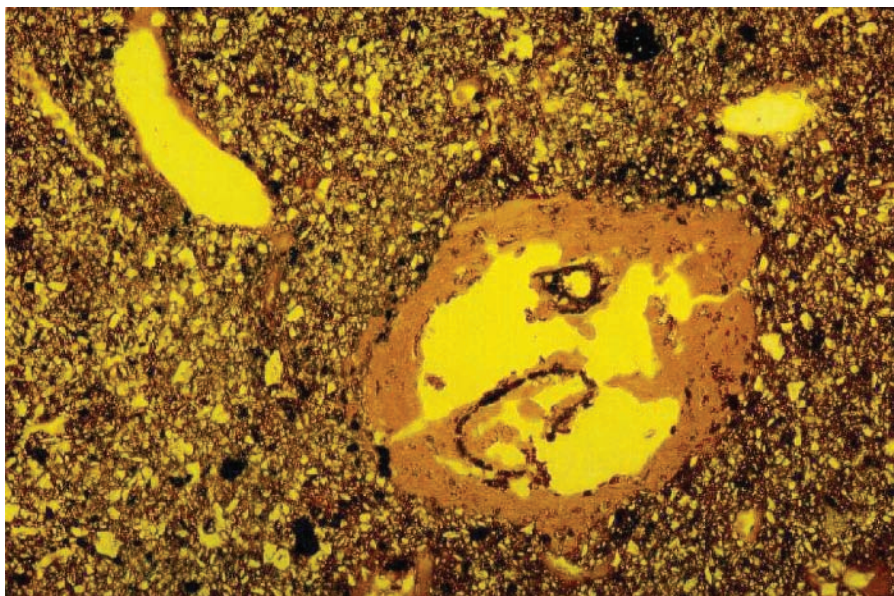


FIGURE 3.2 Orthic Luvisol from Loess, BtC-horizon, depth 90 cm, thin section, bright field, natural width 3.6 mm.

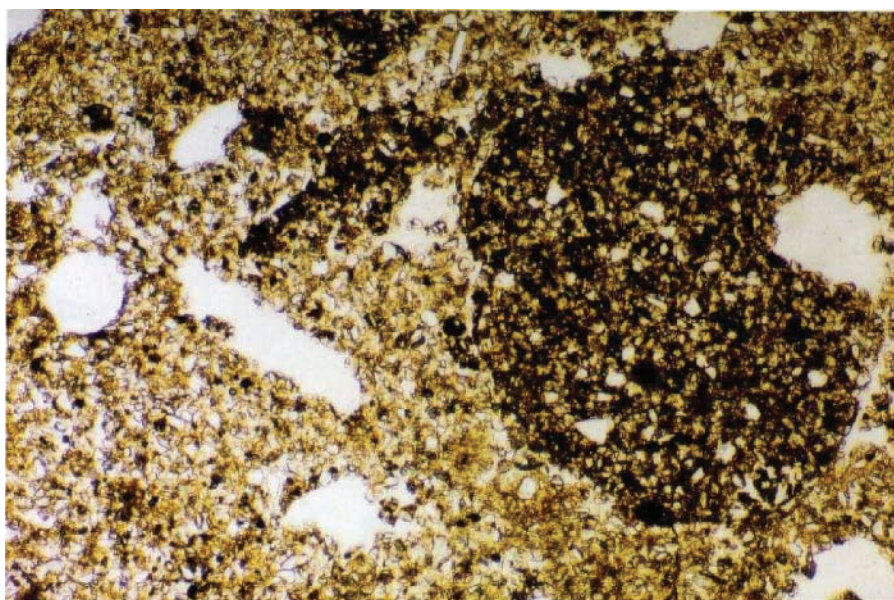


FIGURE 3.3 Tschernosem, AhC-horizon, depth 50 cm, thin section, bright field, natural width 3.6 mm.

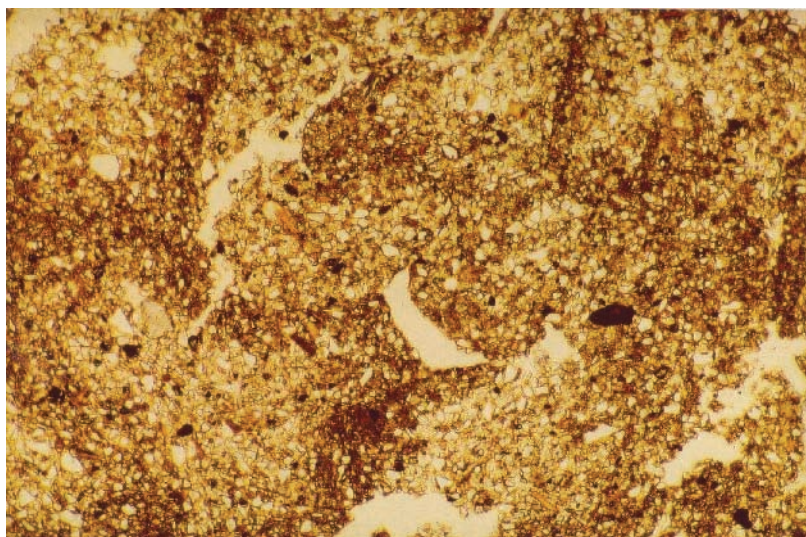


FIGURE 3.4 Tschernosem, Ap horizon, depth 8 cm, thin section, bright field, natural width 3.6 mm

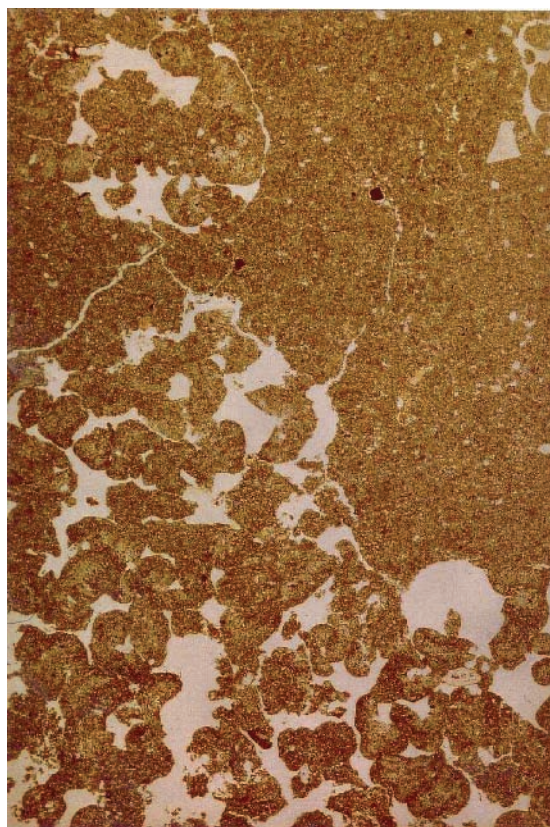


FIGURE 3.5 Tschernosem, Ap-horizon, depth 40 cm, thin section, bright field, natural width 30 mm.

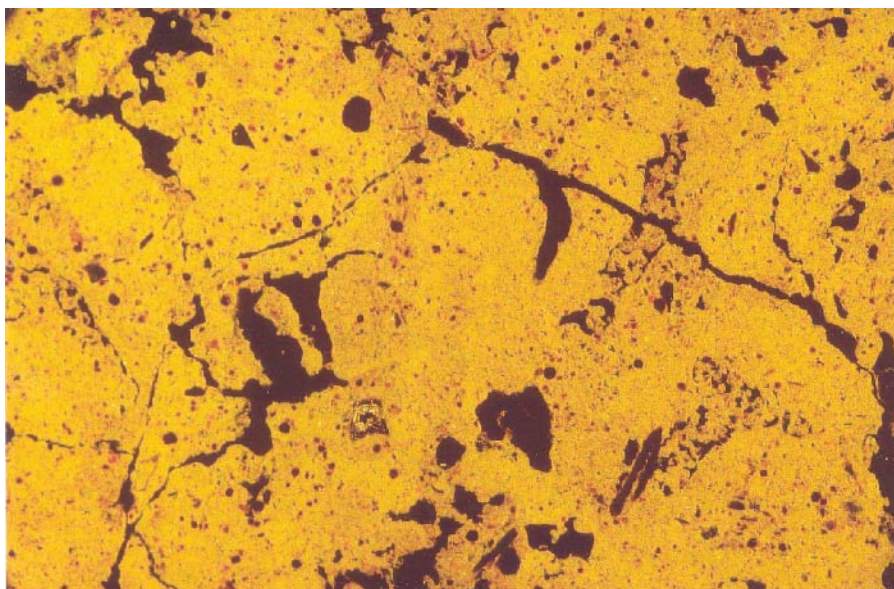


FIGURE 3.6 Terra Fusca, AhBv-horizon, depth 3 cm, thin section, dark field, natural width 9 mm.

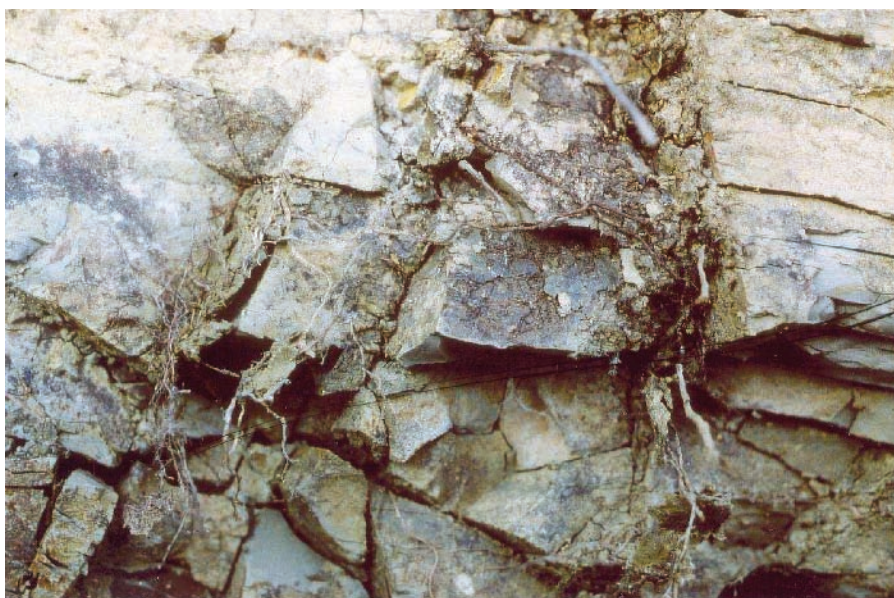


FIGURE 3.8 Triassic marl clay, Cv horizon, depth 60 cm, natural width 10 cm.



FIGURE 3.9 Solifluction cover (clayey silt) on weathered marl clay, natural height 0.7 m.



FIGURE 3.10 Haplic Calcisol, France, natural width 0.4 m.

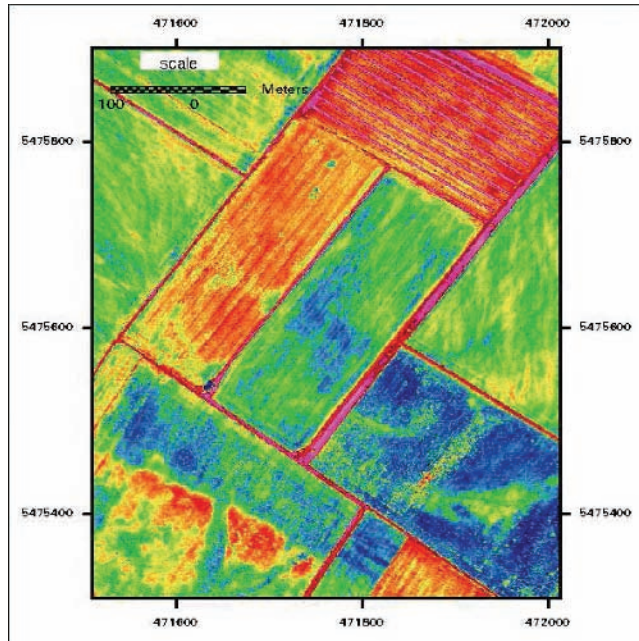


FIGURE 3.12 Air photograph of surface temperature, Rhein valley, Heidelberg, natural width 0.5 km.

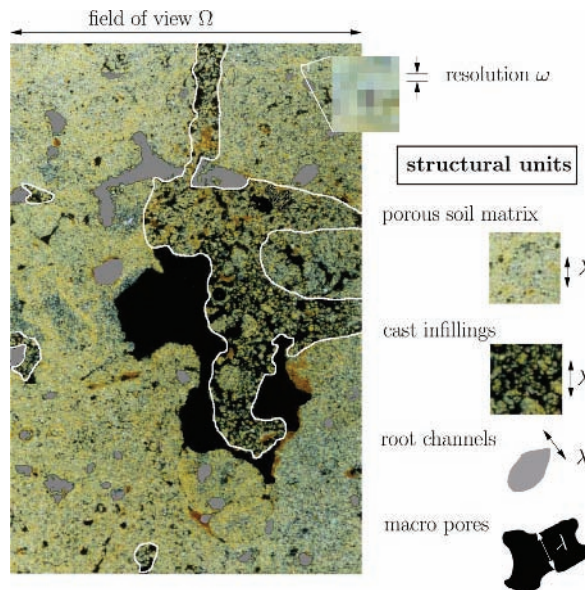


FIGURE 3.14 Vertical section through the subsoil of a Tschernosem (same profile as figure 3.3, figure 3.4, figure 3.5, C-horizon, thin section, incident light, depth 70 cm, natural height 13.5 mm). Observation scale Ω and resolution ω are related to the instrument used to record the image. The different structural units are distinguished by a specific texture as indicated in the right column. They have a specific minimum characteristic length λ . The REV-length Λ of the structural units cannot be shown since $\Lambda > \Omega$. (See text for a detailed explanation.)